# Design of an optimised fed-batch process for insulin precursor production in *Pichia pastoris*

By

**Vinayak BHARDWAJ**




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**(Chemical Engineering)**

Bioprocess Engineering Research Centre

Department of Chemical Engineering

UNIVERSITY OF CAPE TOWN

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# Abstract

The increasing prevalence of diabetes worldwide has greatly increased the demand for insulin, a key type of treatment for many diabetics. The market for insulin has increased by USD 4 billion over the last five years and is set to continue rising. There is thus a renewed interest in increasing the global insulin supply. For this purpose, the methylotrophic yeast *Pichia pastoris* has emerged as an additional microbial host for recombinant insulin production.

A genetically modified *Pichia pastoris* Mut$^S$ strain, engineered to produce the insulin precursor, was used as the experimental system in this study in order to optimise the insulin production process. The experimental system developed in this study employed a two-stage fed-batch feeding strategy in which growth was optimised by feeding glycerol to boost biomass followed by induction of the gene encoding insulin precursor by feeding methanol. Fed-batch conditions studied (media, carbon source, methanol induction conditions) were informed by a series of shake flask experiments. Despite higher biomass yields on glucose compared to glycerol on both chemically defined and complex media in shake flask experiments, the production of ethanol during growth on glucose possibly due to overflow metabolism made it an unfavourable carbon source for large-scale production of insulin precursor. This was owing to the ethanol repression of the alcohol oxidase 1 (AOX 1) promoter of *P. pastoris*.

Batch bioreactor experiments on glycerol in complex and chemically defined media revealed key differences in glycerol uptake, especially at the start of the experiment. The higher biomass production and substrate utilisation in the chemically defined medium over the complex medium resulted in its preferred use for fed-batch experiments.

The effect of the residual methanol concentration on protease activity determined in shake flask experiments showed increasing protease activity as residual methanol concentration increased. However, the actual protease activity released was negligible (0.076 Units/mg in the presence of 40 g/L methanol) especially when compared with that observed in the literature. The effect of methanol on growth was insignificant, possibly due to the use of a Mut$^S$ strain which has depleted methanol utilising abilities may have contributed to the poor methanol metabolism observed in shake flasks. Above residual concentrations of 10 g/L methanol, however, cells grew slower than in the control flasks without methanol added.

Fed-batch experiments were designed based on conditions determined in batch shake flask and bioreactor experiments. Following two preliminary runs in which fed-batch conditions were further optimised, three fed-batch runs were conducted at three preset glycerol feed rates, two of which were at 0.17 /h and the final one was at 0.21 /h. The glycerol feeding rate was controlled to prevent accumulation of glycerol in the reactor. The biomass increase in each of the fed-batch runs was 148, 152 and 110 g in total, respectively, as the reactor volume was increased from approximately 2.5 to 5 L. Methanol was added during the induction phase at 3.8 g/Lh,2.8 g/Lh and 2.94 g/Lh respectively, resulting in growth rates of 0.006 /h, 0.002 /h and 0.005 /h respectively. No residual methanol was present during the induction phase.

Following the optimisation of biomass productivity, efforts to detect and quantify insulin employed a western blot, HPLC and MALDI techniques. These methods were unsuccessful in detecting insulin precursor in the cell lysate or in the extracellular mileu. MALDI MS analyses revealed the presence of ubiquitin and protease in the extracellular matrix, suggesting that the insulin precursor may have been degraded upon secretion. A putative mechanism of ubiquitin-mediated proteolytic degradation of the insulin precursor is presented. In addition, errors in translation may have resulted in the lack of insulin precursor production.

# Declaration

I know the meaning of plagiarism and declare that all the work in the document, save for that which is properly acknowledged, is my own.

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Vinayak Bhardwaj (BHRVIN001)

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# List of Abbreviations

| | |
|---|---|
| AOX | Alcohol Oxidase |
| CDW | Cell Dry Weight |
| CDM | Chemically defined medium |
| $DO/dO_2$ | Dissolved oxygen |
| FM 22 | Cultivation Medium 22 (chemically defined) |
| F | Flow rate (volume/unit time) |
| FDA | Food and Drug Administration |
| G | Glycerol |
| GC | Guanine/Cytosine |
| IDF | International Diabetes Federation |
| $Mut^S$ | Methanol Utilisation Slow |
| $Mut^+$ | Methanol Utilisation Plus |
| $Mut^-$ | Methanol Utilisation Deficient |
| PTM | Pichia Trace Metals |
| RP-HPLC | Reverse Phase High Pressure Liquid Chromatography |
| SCP | Single Cell Protein |
| v/v | volume of solute/volume of solvent |
| µ-stat | constant growth rate |

# Nomenclature

| | |
|---|---|
| D | dilution rate (/h) |
| dS/dt | rate of change in substrate concentration (g/Lh) |
| dX/dt | rate of biomass formation (g/Lh) |
| F | Flow rate (volume/unit time) |
| $G_0$ | Initial glycerol concentration (g/L) |
| $K_S$ | Monod half-rate saturation constant (g/L) |
| P | Product concentration (g/L) |
| S | substrate concentration (g/L) |
| $S_0$ | initial substrate concentration (g/L) |
| t | time (hours) |
| $\mu$ | specific growth rate (/h) |
| $\mu_{max}$ | maximum specific growth rate (/h) |
| V | volume (L) |
| $V_0$ | initial volume (L) |
| X | biomass concentration (g/L) |

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# Chapter 1

# Introduction

### 1.1 Background

Diabetes is a condition characterized by impaired glucose metabolism, caused by defects in the insulin biosynthetic pathway. Chronic, incurable accumulation of glucose in the bloodstream may ultimately result in multiple organ damage and failure. The World Health Organization (WHO) estimated in 2011 that approximately 2.9 million deaths per annum are attributable to diabetes. Due to demographic changes and increasing urbanization it has been estimated that the current population of diabetics (171 million) will double by 2030 (Zhang *et al.* 2010). A substantial proportion of this population would require a regular supply of insulin.

Insulin is a 51-amino acid peptide that is crucial for glucose homeostasis and therefore useful in the treatment of diabetes (Wang *et al.* 2001). Insulin was the first recombinant product to gain regulatory approval from the Food and Drug Administration authority (FDA) in the US. Since then it has become widely used in the treatment of type I and is increasingly used in the treatment of advanced forms of type II diabetes. The current value of the insulin market worldwide (most of which is recombinant-based) is estimated to be USD 16 billion, having increased by approximately USD8 billion since 2006 (Novo Nordisk, 2011). The major recombinant vectors used include the bacterium *E. coli* and the yeast organism *Saccharomycescerevisiae.* In *Escherichia coli,* recombinant insulin is packaged in inclusion bodies inside the cell. Insulin produced in *S. cerevisiae* is manufactured as a precursor secreted out of the cell. Both methods are commercially feasible. Recent research has investigated the possibility of using alternative yeast species to *S. cerevisiae* largely due to its weakly inducible promoter. The most promising alternative appears to be the yeast *Pichia pastoris* (Porro *et al*. 2010).

*P. pastoris* is a methylotrophic organism capable of subsisting upon methanol as a carbon source. Its strongly inducible alcohol oxidase 1 (AOX 1) promoter and its capacity to reach high cell densities by relatively simple cultivation strategies make it a favorable vehicle for high-level and stable protein production (Cregg 1999). The capacity of *P. pastoris* to produce insulin precursor has been demonstrated in numerous studies on both small and large-scale despite the yield achieved across

each study being highly variable(Porro *et al.* 2010). Molecular factors affecting the expression level of a recombinant protein in *P. pastoris* include its amino acid sequence, structure, site of expression and number of gene copies encoding it. Additionally, recombinant protein expression is affected by the cultivation strategy used for production (Pais-Chanfrau *et al.* 2004, Gurramkonda *et al.* 2010, Mansur *et al.* 2005a). This study is primarily focused on optimizing biomass production with a view to ultimately enhancing insulin precursor expression.

### 1.2 Focus of this study

The overall aim of this study was to enhance insulin production in *P. pastoris.* This required both the production of a high cell concentration cell culture at optimal biomass productivity as well as reliable and efficient insulin production. For high cell concentration, ideally we desire high biomass concentration for efficient use of reactor volume, high cell productivity to maximise the space-time utilisation, efficient substrate utilisation to minimise input costs and selection of a substrate that minimised protease release. Therefore, this study investigated the growth behaviour of *P. pastoris* in order to optimise the reaction conditions for greatest biomass production. To do this, a series of shake flask and bioreactor experiments were performed using two media types (complex and chemically defined) and three different carbon sources (glucose, glycerol and methanol). By identifying and formalizing trends in the behaviour of the system during batch growth into a simple model describing biomass production, a substrate feeding strategy was developed.

In contrast to previous approaches, this study is unique in that it attempts to enhance the system as a whole rather than narrowly focussing on increasing the expression level. This approach is superior because it simplifies attempts to scale up the process. Additionally, the same approach can be applied to expression of other recombinant products in *P. pastoris.* This study is motivated by the need to increase the global insulin supply to meet the growing demand, particularly within sub-Saharan Africa.



### 1.3 Thesis structure

**Chapter 1** provides the background and rationale for this study **Chapter 2** presents a review of the literature on current recombinant methods of insulin production as well as a detailed examination of *P. pastoris* as a recombinant vector. The comprehensive overview of its role in insulin production highlights limitations in existing research into *P. pastoris* as a recombinant vector and the

opportunities these present for further investigation. These opportunities form the basis for this research.

**Chapter 3** delineates specifically the research methodology employed in the achievement of these research objectives. It further details the specific research approach adopted in this study.

**Chapter 4** presents the results obtained in the process optimisation experiments. The chapter describes the first set of shake flask experiments conducted for the purpose of identifying the best carbon source for large-scale optimisation. Subsequently, the fed-batch conditions were optimised for the three major stages of *P. pastoris* cultivation, namely: biomass production in batch phase, exponential biomass cultivation in the fed-batch phase and methanol induction in the final methanol induction phase. Each of the parameters required to develop this final three-phase fed-batch process was investigated under batch conditions in shake flask and bioreactor studies prior to the final fed-batch experiment. The final set of fed-batch experiments was designed on the basis of these data. Finally, the attempts to identify insulin precursor are presented.

**Chapter 5** presents a summary of the key conclusions of this thesis and posits recommendations for future inquiry into the optimisation of insulin production.

# Chapter 2

# Literature Review

### 2.1 Insulin as a key therapeutic in the treatment of diabetes

Insulin is a polypeptide hormone that consists of two peptide chains, the A and B chains linked by two disulfide bonds. The A chain consists of 21 amino acids and contains an internal disulphide bond. The B chain consists of the remaining 30 amino acids (as shown in Figure 2.1 below) and is cross-linked to chain A by two disulphide bonds. All vertebrates produce insulin and the structure is similar in these species: for instance, the difference between porcine and human insulin is merely a single amino acid. Insulin plays a crucial physiological role in the regulation of sugar in the body, fatty acid synthesis, formation of triglycerides and amino acid synthesis (Robinson 1953). Insulin binds to cognate receptors present on cell surface membranes and triggers intracellular biochemical changes which allow cells to take up glucose present in the bloodstream. Severe disruptions in the insulin production cascade are associated with the condition known as diabetes. Diabetes is characterized by excessive blood glucose levels (hyperglycemia) and the inability of cells to absorb glucose, depriving them of energy. Thus diabetic individuals secrete large amounts of glucose in urine (Himsworth 1949) .



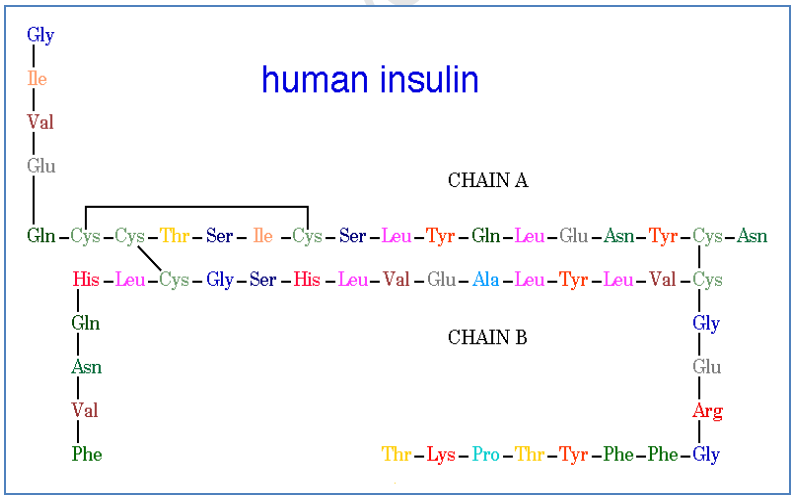


**Figure 2.1: Insulin molecule showing A and B chain linkages** (Pesterfield 2009)

There are two major types of diabetes. **Type 1 diabetes**, called insulin-dependent diabetes mellitus occurs when individuals fail to produce sufficient amounts of insulin. Mainly affecting youths under 20, this is the less prevalent form (10% of diabetics carry this form). **Type II diabetes**, the most prevalent form, is the insulin-independent form of diabetes. It is characterized by the body's inability to produce insulin and/or the body's resistance towards insulin (Schinner *et al.* 2005).

Type II diabetes is controlled mainly by changes in lifestyle and may be treated with insulin injections at an advanced stage (Gale 2001). Type I diabetes is controlled by regular injections of insulin and strict control of diet. The administration of insulin as a treatment for diabetes has an extensive history. Banting *et al.* (1922) reported the first successful treatment of a human patient with an insulin preparation obtained from animal pancreatic extract. Subsequently a recombinant method was developed in *E. coli*.

According to estimates from the International Diabetes Federation, the number of diabetics is expected to increase by 54%, from approximately 285 million (2010) to 438 million in 2030. The projected increase for sub-Saharan Africa by 2030 is 98%, from 12.1 million in 2010 to 23.9 million in 2030. The population of individuals characterised as having decreased glucose tolerance in sub-Saharan Africa is expected to rise by up to 75.8% from 26.9 million in 2010 to 47.3 million in 2030 (Shaw, Sicree & Zimmet 2010).

### 2.2 Current market demand for Insulin

According to the International Diabetes Foundation and the Diabetes Atlas, only 50 % of all diabetics worldwide are accurately diagnosed and of this population less than half have access to any diabetes care. Insulin therapy is essential to the survival of type I diabetics (who cannot produce any insulin) and to control the symptoms and progression of a fraction of those with the more prevalent type II diabetes. The average daily insulin requirement is approximately 1.4-2.1 mg (equivalent to 40-60 Units of insulin activity). This amounts to an annual worldwide demand for insulin of about 100 000kg(Walsh 2005).The global market demand for insulin was estimated to be USD 4.5 billion in 2002 and is estimated to be approximately USD 15.4 billion for 2011 (Novo Nordisk 2011). The major producers include Novo Nordisk, Eli Lilly and Sanofi Aventis.

In Africa, the cost of insulin constitutes up to a third of a diabetic patient's annual medical fees. Thus by increasing the supply of insulin, its overall demand might be met at a lower price (Beran, Yudkin 2006).

## 2.3 **Major recombinant systems used to produce insulin**

### 2.3.1 Introduction

Traditionally, industrial applications of biotechnology used eukaryotic and bacterial systems that were naturally producing important primary or secondary metabolites, enzymes or biopharmaceutical products as production platforms. With the advent of recombinant DNA technology, the introduction of entirely novel traits became possible, thus allowing non-natural compounds to be produced in micro-organisms (Schmidt 2004). Recombinant protein production is a multibillion dollar industry with global sales of biopharmaceuticals in particular reaching USD 87 billion in 2008 and projected to increase to USD 169 billion by 2014. Given the high number of individuals requiring injections of insulin (1.5 million individuals in US in 1978), the available porcine and bovine-derived insulin proved insufficient(Genentech 1978). A brief overview of the major recombinant vehicles for insulin production is presented in the following sections.

### 2.3.2 *Escherichia coli*

A series of technological advances led to the production of recombinant insulin as the first recombinant protein. Following the complete sequencing of the human insulin encoding gene, scientists at Genentech inserted insulin-encoding genes that produced A and B chains of human insulin into circular pieces of DNA known as plasmids. The plasmids containing genes encoding A and B chain production were then incorporated into the bacterium *Escherichia coli*. Upon induction of the genes, the A and B chains were produced separately and joined together to form the insulin molecule. A method for producing *proinsulin,* the precursor to insulin, was also developed in *E. coli* (Gross *et al.* 1989). The *E. coli* system is well characterised; it reproduces asexually producing high quantities of biomass in a short space of time, has naturally occurring, easily manipulated plasmids and has higher average specific productivity. However, it is not ideal for eukaryotic protein synthesis as it is incapable of post-translational modifications. Using the two-chain method is costly as it requires additional purification and re-formulation steps which add to the complexity and cost of the process. Despite the availability of proinsulin secreting *E. coli* systems, the bacterium secretes a range of other proteins with implications for downstream purification of insulin from the protein mixture. Further, the higher productivity of bacterial systems is associated with higher oxygen demand. If not met, the formation of by-products results (Porro *et al.* 2010). Currently, *E. coli* is used for the production of insulin by Eli Lilly, where production figures available for the first quarter of 2011 (products known as Humulin and Humalog) show annual sales of USD 897 million (Eli Lilly 2011).

### 2.3.3*Saccharomyces cerevisiae*

Generally bacteria are useful for high-level intracellular production of less complex proteins, as secretory production of more complex protein is usually associated with yeasts (Scott, Brierley & Howland 1995). While the averaged specific productivity is higher in bacteria than in yeasts, yeasts produce a higher biomass. The space-time yields in yeast are therefore higher (Maurer *et al.* 2006). *S. cerevisiae* in particular is attractive for proinsulin secretion because it secretes very few of its own proteins, thus simplifying downstream purification (Ladisch, Kohlmann 1992). In*S.cerevisiae,* the secretion system, folding and proteolytic processing of proteins as well as glycosylation occur in a similar manner to the mammalian system (Moir, Dumais 1987). This is advantageous for expression of eukaryotic proteins (Gleeson *et al.*1998). It does not produce toxic compounds and its widespread use in food biotechnology and beer production has made it an ideal host for production of therapeutic proteins such as insulin and its analogs (Romanos, Scorer & Clare 1992). Expression of proinsulin entails insertion of a DNA fragment encoding a proinsulin-like molecule flanked with a secretion signal that enables export of synthesized insulin. However, often production of heterologous protein occurs during the growth phase, thus limiting maximum protein production due to hindered growth as carbon is diverted towards both biomass production and product formation. Inducible promoters in *S. cerevisiae* have not been effective due to the instability of the plasmid. Often, heterologous proteins are hyper-glycosylated leading to increased immunogenicity, thus complicating regulatory approval as well as diminishing the activity of the recombinant protein (Porro *et al.* 2010). Quality assurance of therapeutic proteins has also become a critical factor in evaluating recombinant pharmaceutical production. A study by Holz *et al.* (2003) compared the expression of over 300 human proteins in *E. coli* and *S. cerevisiae*. Out of these 58 % were intracellularly expressed in *S. cerevisiae* while only 39% were expressed in *E. coli*. NovoNordisk currently uses the *S. cerevisiae* system to produce over half the world's insulin supply (Heinzle, *et al.* 2006).

### 2.3.4 *Pichia pastoris*

As an alternative vehicle for insulin expression, the potential of the yeast *Pichia pastoris* is being explored. It has a strong inducible promoter, can be grown under conditions similar to *S. cerevisiae,* and secretes only a few of its own proteins. Predominantly used as a research tool, commercial insulin production in *P.pastoris* has recently been reported by the Indian company Biocon (Biocon 2011a).

## 2.4 *P. pastoris* as an expression system for recombinant proteins

The discovery that certain yeast species could utilise methanol as a sole source of carbon and energy was viewed with interest by researchers aiming to exploit these organisms for generating yeast biomass or single-cell protein (SCP) for use as animal feed. Researchers studied methanol metabolism in *P. pastoris,* identifying alcohol oxidase (AOX) as responsible for the first step in the enzymatic breakdown of methanol, apparently representing 25-30% of the total soluble protein in *P. pastoris* in cells grown on methanol while being completely absent in cells grown on other carbon sources. Alcohol oxidase is encoded by two genes, AOX 1 and the weakly transcribed AOX 2 (Cregg *et al.* 1999). AOX 1 is responsible for up to 80% alcohol oxidase activity in methanol-induced cells, with the AOX 1 transcript representing up to 5% of total mRNA in methanol-induced cells, while remaining undetectable in non-induced cells. These discoveries led to the isolation of the strongly induced and tightly-regulated alcohol oxidase promoter (Ellis *et al.* 1985). The molecular techniques typically used for *S. cerevisiae* were adapted for use in *P. pastoris* by Cregg and co-workers (1985) to produce the first recombinant expression system in *P. pastoris*. Their system was based on the complementation of a histidonol-dehydrogenase 4 deficient (*HIS4*-deficient) auxotrophic mutant by a plasmid-based HIS4 gene. Tschopp and co-workers (1987) subsequently developed a system to express the first methanol-regulated expression of a heterologous product, the *E. coli* protein β-galactosidase using theAOX 1 gene as its promoter. The system involved induction of AOX 1 gene expression by the addition of methanol to the medium. Additionally, the carbon source present (i.e. glucose) had to be removed in order for de-repression to occur. This discovery has led to the widespread use of *P. pastoris* as a recombinant expression system of choice for a wide variety of proteins (Tschopp *et al.* 1987). To date over 500 recombinant proteins have been expressed in *P. pastoris* with expression levels as high as 80% of total secreted proteins or up to 30% of total cell protein (Plantz *et al.* 2006).



### 2.4.1 Methanol metabolism in *Pichia pastoris*

The methanol metabolic pathway is shown in Figure 2.2. Methanol is used as a carbon and energy source via simultaneous assimilatory and dissimilatory pathways. In the first step of the methanol oxidation pathway, methanol is degraded to form formaldehyde and hydrogen peroxide as by-product. This step is catalyzed by alcohol oxidase and takes place in the peroxisome, thus circumventing hydrogen peroxide toxicity. Subsequent reactions of the methanol oxidation pathway take place in the cytosol (Macauley-Patrick *et al.* 2005). Control of the first step is by carbon catabolite repression and inactivation by glucose or glycerol with de-repression following depletion of the carbon source and induction by methanol.

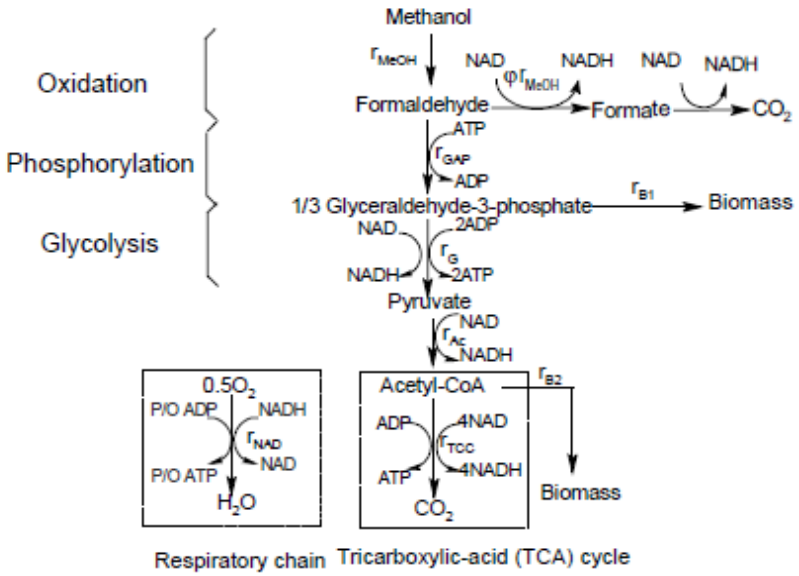


**Figure 2.2 Methanol metabolic pathway in *Pichia pastoris*.**

Diagram adapted from Gleeson *et al.*(1998). Methanol enters the peroxisome and is oxidized by alcohol oxidase to formaldehyde by AOX1. Formaldehyde either enters dissimilatory pathway to produce carbon dioxide or enters the assimilatory pathway to produce biomass (Gleeson *et al.* 1998).

There are three phenotypes of *P. pastoris* host strains and these are classified based on their methanol utilisation characteristics as $Mut^+$, $Mut^S$ or $Mut^-$. The most prevalent forms are the $Mut^+$ and $Mut^S$ strains. The $Mut^+$ strain grows on methanol at the 'wild' type rate as both genes AOX 1 and 2 are present. These strains require a high methanol feeding rate (Daly, Hearn 2005). The Methanol Utilisation Slow ($Mut^S$) phenotype has a disruption in the AOX1 gene; hence it depends on the weaker AOX2 gene for methanol metabolism, resulting in a slower growing and slower methanol utilising strain (Cregg *et al.* 1985).The AOX 2 gene yields 10-20 times less alcohol oxidase activity than the AOX 1 gene (Cregg, Madden 1988). Most heterologous protein expression in *P. pastoris* relies on induction of methanol metabolism when cells are grown on methanol (Veenhuis, Van Dijken & Harder 1983). AOX 1 is the most widely used promoter system and the $Mut^+$ strain is the most popular. It is generally associated with tight regulation, high levels of protein expression and ease of induction. However, it requires high methanol concentrations and constant monitoring of methanol levels (Daly, Hearn 2005). The $Mut^S$ strain requires lower methanol quantities for induction of expression, which is useful for industrial scale manufacture (Macauley-Patrick *et al.* 2005). Further, despite the diminished growth rate compared to $Mut^+$, the final concentration of functional product protein produced by $Mut^S$ strains is occasionally higher as a result of the proper post-translational modifications (Chen *et al.* 2007).

## 2.5 Co-metabolism of methanol with another carbon source

Methanol is inhibitory to growth at high concentrations. Additionally, it produces a low cell yield and its metabolism requires a large amount of oxygen. Thus methanol is typically added as an inducer of AOX1 and AOX2 after biomass production on glycerol (Macauley-Patrick *et al.* 2005).Egli and co-workers (1982) showed that the alcohol oxidase promoter is repressed by the growth substrate, e.g glucose, glycerol and ethanol. Additionally they found that it is possible for the yeast to simultaneously consume methanol and another growth substrate, provided that the second substrate's concentration was kept below repressive levels(Egli, Kappeli & Fiechter 1982).Mixed feeding strategies have also been explored. Growth of *P. pastoris* on glycerol, while maintaining induction of the methanol oxidation pathway has been reported (Siegel, Brierley 1989). The repressive effect of glycerol on AOX1 noted by Brierley (1988) made this a difficult process to implement. As a result, sorbitol has been suggested as a co-substrate with methanol. Celik and co-workers (2009) reported a higher biomass and recombinant protein yield, a lower oxygen utilisation rate and a higher specific AOX activity (Celik, *et al.* 2009). Thorpe *et al.*(1999) compared the sorbitol/methanol, system to glycerol/methanol. While they reported a lower cell yield with sorbitol/methanol a higher specific rate of product formation and a reduced repressive effect of residual sorbitol was noted (Thorpe, *et al.* 1999). The mechanism involved in gene repression based on the specific carbon compound used for growth is not entirely known.

## 2.6 Summary of the major factors making *Pichia pastoris* an ideal recombinant system for protein expression

A number of critical factors have led to *P. pastoris* becoming the vehicle of choice for this study. These factors are listed below.

I. The promoter controlling transcription of the AOX gene is strongly and tightly regulated, resulting in the recombinant protein constituting up to 35% of the total cell protein from a single encoding gene. This promoter is controlled through induction by methanol and repression in the presence of specific alternative substrates (e.g glucose and glycerol). This limits the proliferation of low or non-expressing strains during growth. Methanol is an inexpensive inducer and provides a feasible source of carbon and energy during fed-batch cultivation.

II. The cultivation protocols for *P. pastoris* are well-developed and designed for high productivity, large-scale cultivation of up to 100L. The chemically defined media and trace

metals mixture is inexpensive and has been developed for a number of carbon and energy sources including methanol.

III. Screening transformed mutants for large scale protein manufacture is made easier by the fact that methods developed in *S. cerevisiae,* e.g. mutant detection using markers such as *HIS4*complementing the auxotrophic strain are adaptable to *P. pastoris.* The dominant antibiotic resistant marker G418 which confers resistant to geneticin, make cloning, gene targeting and increasing of copy number easier in *P. pastoris*.

IV. Generally, *P. pastoris* is a poor fermenter and it does not accumulate ethanol rapidly, thereby preventing the repressive effect of ethanol on the AOX promoter.

V. *P. pastoris* produces high yields of recombinant proteins, is capable of many eukaryotic post-translational modifications, e.g. glycosylation, and, due to its capacity for protein secretion (using the *S. cerevisiae* secretion signal), downstream purification is easier.

VI. It is a food-grade yeast which generally simplifies regulatory approval of *Pichia* products.

VII. Despite the attractive qualities of *P. pastoris,* protein yields are known to be highly variable across recombinant strains. Essentially, this may be due to molecular or cultivation-related factors.

### 2.7 Cell level strategies to optimise recombinant protein production in *P.pastoris*

Broadly speaking protein production can be optimised by manipulating the cell genome or by improving the cultivation techniques. The following discussion presents some of the key cell-level strategies to enhance protein production in *P. pastoris*.

*Codon usage:* the specific base sequence of the gene cloned into the plasmid must resemble the original gene sequence in order to enhance the production of the desired protein.

*Guanine/Cytosine (GC) levels:* The GC content of the plasmid determines its stability as well as the fidelity of replication. Thus for optimal gene expression a high GC content is ideal. Optimizing the GC content of a cloned human glucocerebrosidase gene led to a 10.6-fold increase in protein expression levels while codon-optimisation increased protein 7.5-fold, thus highlighting the value of such approaches (Sinclair, Choy 2002).

*Protease-deficient strains:* In optimizing heterologous protein expression, avoiding proteolysis is a major concern. The causes of proteolysis in high cell concentration cultures are discussed in Section

2.9.2. Molecular methods to circumvent this include the approach of Boehm *et al.* (1999) who were able to decrease the proteolytic degradation of C-terminal end of murine endostatin by disrupting the gene encoding a major cellular protease, thus increasing the production of intact protein. However, these protease-deficient strains are not as fast-growing as wild type strains and are difficult to transform (Potvin, Ahmad & Zhang 2010).

*Multicopy Expression:* It has been reported that when heterologous genes were under the direction of the AOX or the glyceraldehyde phosphate (GAP) promoter, increases in the number of copies of the gene led to an increase in protein. For example, increasing the number of copies of the gene encoding hepatitis B surface antigen from one to four led to a four-fold increase in protein (Romanos *et al.* 1991). However, several researchers have observed no benefit in increasing the gene copy number (Hohenblum *et al.* 2004). This may be due to the increased burden on the cell's energy reserves that results from transcribing and translating the extra genes (Macauley-Patrick *et al.* 2005). In particular, rate-limiting steps have been suggested to be due to post-translational effects such as folding in the endoplasmic reticulum, membrane translocation and signal sequence processing (Hohenblum *et al.* 2004).

*Mut Phenotype:*The $Mut^S$ strain typically requires long induction periods of up to a maximum of 100 hours due to its slow growth on methanol but is known to produce high quantities of intracellular proteins (Romanos, Scorer & Clare 1992). $Mut^+$ strains grown in fed-batch cultures require less induction time (as little as 50 h), resulting in a higher productivity. However, methanol results in poor growth, requires high quantities of oxygen and evolves considerable amounts of heat (Shay*et al.* 1987). Furthermore the $Mut^+$ strainis highly sensitive to high residual methanol concentrations which result in a loss of AOX1 activity and cell death (Brierley *et al.* 1990b).

## 2.8 Strategies for large-scale high cell concentration cultivation of recombinant *P.pastoris*

The earliest cultivation procedures for *P. pastoris* were developed by Wegner in 1983. This provided a reference for Brierley and co-workers who reported a process protocol for $Mut^S$ and $Mut^+$ strains producing up to 600 mg/L of enzyme (Brierley *et al.* 1990a).

Protein expression in *P. pastoris* generally entails three steps: the glycerol batch phase, the glycerol fed-batch phase and the methanol induction phase. Cells are grown on glycerol in batch phase until glycerol in the medium is exhausted. In the second phase, the glycerol fed-batch phase biomass is increased to a predetermined concentration prior to induction by supplying glycerol in a growth-limiting fashion. By preventing the accumulation of glycerol, its repressive effect on the AOX1 promoter is removed. In order to trigger protein expression, the final methanol induction phase is

required. Methanol is fed at a limited rate or maintained at a predetermined rate (Brierley *et al.* 1990a). This induces expression of the alcohol oxidase gene leading to methanol utilisation and recombinant gene expression.

### 2.8.1 Glycerol batch and fed-batch phase followed by methanol induction phase

The purpose of the glycerol batch and fed-batch phases (GBP and GFP) is to optimise biomass production before inducing protein expression. The strategies to run GBP and GFP are the same across the various *P. pastoris* phenotypes $Mut^+$, $Mut^S$ and $Mut^-$, since their growth on glycerol is similar (Chiruvolu *et al.* 1998). Brierley *et al.* (1990a) have shown that a glycerol concentration beyond 40 g/L results in 0.5-2.4% ethanol production which represses the AOX promoter. The glycerol fed-batch phase is designed to maintain a desired specific growth rate. Glycerol must be fed at a limiting rate to maintain an exponential growth rate and to prevent the accumulation of glycerol which represses the AOX 1 promoter (Zhang*et al* 2000). Overfeeding glycerol may additionally result in ethanol production (due to overflow metabolism) which results in promoter repression (Inan*et al*, 2001b). In the glycerol batch and fed-batch phases, the AOX1 promoter is repressed and no recombinant protein is produced. The length of the glycerol fed-batch phase is determined by the biomass production required prior to methanol induction.

Methanol is added upon glycerol exhaustion to switch on the AOX1 promoter, hence protein expression. The concentration of methanol fed and the overall methanol feeding scheme varies considerably. The $Mut^+$ strain typically uses methanol as a sole carbon and energy source during induction. In these cells, the transition phase lasts 4-5 hours before cells adapt to the alteration in carbon supply. In $Mut^S$ strains however, the overall induction and protein production phase lasts up to 70 hours (Potvin*et al*,2010)

## 2.9 Environmental strategies for improved recombinant protein production

### 2.9.1 Growth media

The best cultivation conditions are often strain dependent and based on the particular heterologous protein being expressed. The most widely used medium for high cell concentration cultivation is the basal salt medium (BSM) prescribed by the *P. pastoris* expression kit manufacturers Invitrogen (Prinz *et al.* 2004). Its unbalanced composition, tendency to form precipitates in the bioreactor and high ionic strength are cited as non-ideal (Cos *et al.* 2006c). Alternative media are proposed by d'Anjou *et al.*(Anjou &Daugulis 2001) and by Stratton *et al.* (1998a), the latter formulated the cultivation medium-22 (FM22). An elemental comparison of the different media is presented in Table 2.2.

**Table 2. 1 Elemental comparison between three different media used in the cultivation of *P. pastoris*. Table adapted from Cos *et al,* 2006** (Cos *et al.* 2006b)

| Element | BSM (g/L) (Prinz *et al.* 2004) | FM22 (g/L) (Stratton, Chiruvolu & Meagher 1998a) | D'Anjou (g/L) (D'Anjou, Daugulis 2001) |
|---|---|---|---|
| *N | $NH_4OH$(pH control) | 1.06 + ($NH_4OH$ pH control) | 4.24 |
| P | 12.27 | 9.76 | 2.73 |
| K | 11.05 | 18.74 | 3.45 |
| Mg | 1.47 | 1.15 | 0.46 |
| Ca | 0.27 | 0.12 | 0.1 |
| S | 5.51 | 5.46 | 5.47 |
| Cl | -- | -- | 0.17 |

*Nitrogen supplied as $(NH_4)_2SO_4$ in D'Anjou *et al.* (2005) protocol

All defined media aresupplemented with a range of trace elements including Fe, Mn, Cu and the vitamin biotin normally used as part of the *Pichia Trace Metals 1* (PTM 1) solution. The precise effect of each of these elements has not been studied in depth, specifically with respect to the $Mut^S$. Efforts to optimise biomass production in chemically defined media have focused mainly on the combination of predetermined vitamin, mineral salts and carbon substrates leading to improved cell growth(Ghosalkar *et al.* 2008).

### 2.9.2 Proteolytic activity and stability of recombinant proteins

As mentioned, proteolysis is a major limitation in the production of recombinant protein during *P. pastoris* cultivation. *P. pastoris* produces proteases whose levels vary according to glucose and nitrogen concentrations in the media (Hansen *et al.* 1977). The causes of proteolytic degradation of recombinant ovine interferon protein are primarily linked to methanol metabolism as well as high pH conditions (Sinha *et al.* 2003). It has also been shown by Sinha and co-workers(2003) that nitrogen availability did not significantly decrease the proteolytic activity of the $Mut^+$ strain and that protease activity was mainly due to the switch from glycerol to methanol. Studies of cell viability showed that methanol metabolism results in $H_2O_2$ production. Upon high $H_2O_2$ accumulation within the cell, lysis occurs, releasing proteases into the extracellular medium. The vacuolar proteases typically detected in the supernatant of *P. pastoris* cultures are proteinases A and B, carboxypeptidase Y and aminopeptidase (Sinha *et al.* 2003). Jahic and co-workers (2003) found that decreasing the pH resulted in an increase in recombinant protein, possibly due to denaturation of proteases. Studies on the effect of methanol and pH on protease activity have been limited to $Mut^+$ strains. Given that $Mut^S$ strains possess a much lower AOX activity, the by-products of methanol

metabolism are likely to be produced at a much lower rate and would hypothetically result in a lower protease expression.

## 2.10 Modes of cultivation

### 2.10.1 Batch cultivation

Microbial cultivation can be carried out in batch, fed-batch and continuous modes. Batch processes are closed systems. The media components required for the process are added to the bioreactor at the beginning of the process. The only material added during the course of batch cultivation is the pH control solution and gaseous mixtures. The system is not at steady state. The pH and temperature may be maintained at constant values. Additionally, the homogeneity of the mixture is enhanced by constant agitation.

Growth and substrate consumption are typically described by the Malthus equation where the specific growth rate depends on substrate availability according to Monod Kinetics:

$$\frac{dX}{dT} = \frac{\mu_{max} .X.S}{K_S + S} \qquad \text{Equation 2.1}$$

$$\frac{dS}{dT} = -\frac{\mu.X.S}{Y_{X/S}} + m_S.X \qquad \text{Equation 2.2}$$

According to the mass balance therefore, the biomass concentration increases as the substrate concentration decreases. Given that the substrate concentration at the end of the cultivation process is close to zero, the yield of biomass on substrate is given by:

$$Y_{x/s}^{overall} = \frac{X_{final} - X_{initial}}{S_0} \text{quation 2.3}$$

The overall yield coefficient is distinct from Yx/s or $(Yxs^{true})^{-1}$ which is the stoichiometric rate between biomass formation and substrate consumption. Where substrate is used primarily for assimilation into biomass with limited consumption for maintenance, these values will be similar.

However, if there is little variation in these rates during batch growth, (if for instance, there is a long exponential phase and a short declining growth phase) the overall yield coefficient would be similar to the 'true' yield coefficient.

### 2.10.2 Monod growth kinetics

To connect the rate of growth to the substrate utilisation profile, the model proposed by Monod was used. This model notes that the rate of growth of a cell culture relative to its maximum growth rate is proportional to the limiting substrate concentration and a constant. The expression for such growth resembles that of Michaelis-Menten enzyme kinetics and is shown in equation 2.4

$$\frac{\mu}{\mu_{max}}\frac{S}{S} = \frac{S}{S+K_S} \qquad \text{Equation2.4}$$

Where μ is the specific growth rate, $\mu_{max}$ is the maximum specific growth rate (units /h), S is the limiting substrate concentration (g/L) and $K_s$ is the substrate concentration at which the specific growth rate is reduced by one half. This value indicates the affinity of the micro-organism for the substrate (Shuler & Kargi 1991; Doran 1995).

### 2.10.3 Fed-Batch cultivation

Fed-batch cultivation was initially devised as a method for achieving high density yeast cultures by providing malt in limiting quantities to prevent an over-accumulation of ethanol, a toxic by-product{{268 McNeil and Harvey 1990}}. The method is useful in producing both growth associated and non-growth associated recombinant proteins in microbial populations. For growth associated product formation, extended growth by controlled supply of the growth-limiting reagent can boost growth, hence product formation, without substrate inhibition or overflow metabolism. For non-growth associated systems, e.g. where product formation is induced, the fed-batch process would have two distinct phases. In the first phase, biomass would be produced; in the second,product would be formed by addition of the carbon source and nutrients required for product formation. This is of particular interest to inducible recombinant production systems such as *P. pastoris*(Doran 1995).

Feeding strategies specific to fed-batch cultivation of *P. pastoris* are presented later in this review. Generally, there are 'feedback' driven strategies and 'non-feedback' strategies that are used to control the fed-batch process. In fed batch processes that operate under feed-back control, cultivation parameters such as pH and dissolved oxygen are constantly monitored. In response to a decrease in either parameter due to nutrient depletion as organisms grow, the 'signal' detected results in the addition of a predetermined volume of nutrient feed to the medium. Given the constant monitoring involved, this method has the advantage of providing sufficient amounts of medium to the system, preventing over-accumulation of the limiting substrate.

There are broadly speaking two different fed-batch processes that can be operated.

These include:

i) Provision of nutrient feed at a constant rate, resulting in a decreasing specific growth rate
ii) Maintenance of a constant specific growth rate of the micro-organism by exponential feed of substrate

A number of assumptions are made before each of these scenarios can be represented mathematically. In particular, it is assumed that:

- No culture is removed from the reactor during the fed-batch process
- The yield on the growth limiting reagent is constant
- The cells remain in a state of 'balanced' growth
- Cell metabolism is not completely dependent on the age of the culture
- Cell maintenance can be ignored
- The increase in volume of the culture is due to the nutrient feed added.



### *i) Constant substrate feeding rate*

This is the simplest feeding method. A predetermined feeding rate is used to add the limiting substrate continually into the bioreactor. As the biomass concentration increases, the feed addition is less than the rate of substrate uptake. The cell growth rate increases until the concentration of limiting substrate cannot support the rate of biomass formation, thereby resulting in a decrease in cell growth rate (Clark and Blanch 1997). As the growth limiting substrate is consumed rapidly by the cells, the specific growth rate of the organism is given by equation 2.5.

$$\boldsymbol{\mu = \frac{Y_{x/s}.S.F}{X.V}} \qquad \text{Equation 2.5}$$

where V is the Volume of the reactor, X is the biomass concentration during fed-batch, $Y_{X/S}$ is the biomass yield coefficient on carbon substrate and F is the rate at which the limiting substrate is fed to the bioreactor. Integrating the above expression gives

$$\int XdX = C \int dt \qquad \text{Equation2.6}$$

where C is a constant term equal to the product of $Y_{X/S}$, S and F (constant terms at quasi-steady state). Integrated over time, the expression above yields equation 2.7 to describe the rate of biomass produced during fed-batch phase

$$XV = \left(Y_{X/S.}\, S.F\right)t + X_0.V_0 \qquad \text{Equation 2.7}$$

The relationship between biomass and time is linear according to equation 2.6

### *ii) Exponential feeding*

When the limiting substrate is fed so as to maintain a specific growth rate of the cells during fed-batch growth, such a feeding scheme is known as exponential feeding.

At t = 0 h, $V=V_0$ and $X = X_0$, the cell mass balance equation can be written as

$$XV = X_0.V_0 e^{\mu t} \qquad \text{Equation 2.8}$$

The feed rate is adjusted to maintain a quasi-steady state such that $\frac{ds}{dt} = 0$

Assuming that $Y_{X/S}$ remains constant, and substituting equation 2.7 into the substrate mass balance, an exponentially increasing substrate feed rate can be determined

$$F = \frac{\mu.X.V}{S_0.Y_{X/S}} = \frac{\mu.X_0.V_0}{S_0.Y_{X/S}}.e^{\mu t} \qquad \text{Equation 2.9}$$

By exponential feeding, a desired specific growth rate can be maintained, acetate and ethanol production can be minimized, and fed-batch process time can be reduced while ensuring an increase in biomass productivity and yield (Clark, Blanch 1997).

Reviews of recombinant systems in general and methods of enhancing large scale protein production (Makrides 1996, Yee and Blanch 1992) identify a number of crucial considerations in the design of a recombinant protein production system:

i. The nutrient composition of the medium must be optimised to ensure that, e.g. non-carbon nutrients that are growth limiting, are added together with the substrate and not in isolation. The medium osmolarity and pH must be optimised as this may affect the cell concentration and yield.

ii. The oxygen transfer rate can be limiting during cultivation and must be optimised to ensure that cells are not oxygen-limited as this may necessitate lower substrate feeding rates.
iii. Ensure that, where possible, inducible promoters are used in order to disaggregate the growth and product formation stages of the cultivation.
iv. During fed-batch cultivation, growth substrate must be fed so as to prevent over- or under-feeding of the cells, either through predictive or feed-back control systems.

The last factor mentioned is particularly essential to the successful development of a fed-batch process.

## 2.11 Control of the substrate feed for recombinant protein production under the AOX1 promoter

### 2.11.1 The dissolved oxygen (DO) 'spike' method

In order to maintain low methanol levels, substrate feeding may be varied to maintain a stable dissolved oxygen concentration (DO-stat method). This strategy is applicable and relevant to controller design in many industrial settings where high cell densities and oxygen transfer limitation are routinely encountered (Stratton*et al.* 1998b).

DO-stat control is used for the addition of reduced carbon substrates depending on the DO concentration in the medium. This approach has two purposes. First, it allows a high cell concentration to be achieved while minimizing the accumulation of incompletely reduced substrates. Secondly, it maintains the active cellular states for prolonged periods of time (Lim *et al.* 2003). During active cell growth, carbon is utilised rapidly and the oxygen demand is high. Thus DO tension drops rapidly. However, upon depletion of the carbon source, the DO tension rises. Further supply of a carbon source reduces the DO levels due to the re-activation of metabolic pathways. Using these phenomena a simplified indirect feedback protocol that couples substrate feeding to measurements of dissolved oxygen tension can be devised. When the DO tension reaches a pre-set value, the carbon source is added until DO falls to a pre-determined value, after which carbon addition is stopped. Thus the concentration of the particular carbon source can be kept under control (Lim *et al.* 2003)

However this method is tedious and subject to fluctuations in DO profiles. To counteract this, a methanol control strategy based on a methanol sensor has been developed by Katakura *et al.* (1998). DO-stat control methods are simple to operate, although not entirely reliable. Methanol metabolism is a high oxygen-utilising process (Jungo *et al.* 2007). During rapid cell growth, when oxygen consumption is high, DO levels are low and, consequently, methanol levels are low if DO-stat

control is exclusively relied on. Thus cells may be starved and protein yields reduced. Conversely, if inhibitory methanol levels are reached, a sharp increase in dissolved oxygen will be observed. The response of the system will be to increase the methanol feeding rate, resulting in accumulation of methanol to toxic levels. Also, where such strategies were used, the methanol concentration and the specific growth rate were not maintained constant and so it is difficult to accurately determine the effect of these parameters on protein production (Potvin*et al.* 2010).

### 2.11.2 Constant methanol concentration

Zhang and co-workers (2000)have similarly proposed a similar sensor-based methanol feeding scheme. This method uses feedback control systems to maintain stable methanol concentrations. The simplest of these employs an 'on-off' control method (Zhang *et al.* 2000, Guarna *et al.* 1997, Wagner *et al.* 1997). It is limited to systems where a linear relationship between methanol provision and protein production exists. However, heterologous protein production in *P. pastoris* is a complex non-linear process and so such a strategy may result in fluctuations about a set point. As an alternative, proportional integral (PI) or proportional integral derivative (PID) control methods have been developed for the effective maintenance of methanol concentrations. The optimal control levels are determined by specifying desired biomass concentration and broth volume (Zhang *et al.* 2003).

### 2.11.3 µ-Stat control fed-batch

This method of control forms the basis for the fed-batch experimental strategy devised for the production of insulin in *Pichia pastoris* in this study. It is a widely used feeding strategy that relies on the growth model on the limiting substrate; in this case *P. pastoris* on methanol. The use of a growth rate on methanol allows the development of a pre-determined substrate feed rate profile for a fed-batch process, thus does not require on-line monitoring of system parameters. The feeding profile is derived from mass balance equations (Cos *et al.* 2006a). Specific growth rate control during the methanol fed-batch phase is an effective strategy for process optimisation as most biochemical processes including protein production, are directly or indirectly linked to growth *et al.* 2003). Maintaining a constant growth rate ensures process reproducibility and facilitates the study of growth-rate related effects on heterologous protein manufacture *et al.* 2010).

Working with $Mut^+$ strains that are able to achieve slightly higher growth rates on methanol than $Mut^S$ strains, Zhang *et al.*(2003) established an unstructured growth model that related specific growth rates, methanol concentrations and specific methanol consumption rates. In order to ensure a constant growth rate during the methanol induction phase, they used a methanol sensor. By

maintaining a preset growth rate, they obtained a quasi-steady state. Having determined the maximum growth rate of their *P. pastoris* strain on methanol, they computed the substrate consumption rate and determined the overall feeding profile based on desired values of growth rate. This allowed a correlation between growth rates and product formation rates (Zhang *et al.* 2003).

Comparison between the different fed-batch techniques is difficult due to the variability across different recombinant proteins being expressed as well as the differences in strains used and plasmids constructed. Nonetheless, a study by Trinh *et al.* (2003) compared three methanol addition strategies for the production of mouse endostatin production in *P. pastoris.* The first two strategies were based on methanol metabolism and the third one was based on a predetermined exponential feeding rate. The first of the two strategies involved supply of methanol based on the rate of methanol uptake and the second involved adjusting methanol supply on the basis of dissolved oxygen concentrations in the reactor. Both these strategies required online monitoring of methanol, adjusting the feed as required. In the third strategy, methanol was fed to maintain a predetermined specific growth rate. All three strategies resulted in the production of comparable amounts of endostatin. However, the latter strategy had the effect of reducing the amount of methanol fed to the reactor, resulting in lower biomass production. Thus the specific rate of product formation was up to two-fold higher than in the other two feeding strategies, thus ensuring a more scalable approach with fewer downstream processing considerations (Trinh*et al*2003).



### 2.12 Strategies employed to produce insulin in *P. pastoris*

Kjeldsen *et al.* (2000) were the first to report the cloning of the insulin precursor gene in *P. pastoris* as well as its successful expression. The authors used a $Mut^{+}$ strain grown in complex media on glycerol and methanol in a shake flask system. Reverse Phase High Performance Liquid Chromatography (RP HPLC) was used to identify and characterize insulin and to confirm that *P. pastoris* has the capacity to express and secrete insulin in a manner that was better than or equal to that of *S. cerevisiae.*

Since then, numerous authors have developed large scale processes in chemically defined media for the cultivation of $Mut^{+/S}$ strains expressing the insulin precursor. Table 2.2 (on page 26) compares the processes for production of insulin precursor reported in the literature. There has been considerable investigation into whether *P. pastoris* strains expressing multiple copies of the insulin precursor gene produced higher quantities of insulin. Zhu *et al.* (2000) generated a range of *P. pastoris* organisms containing multiple copies (from 0 to 52 copies) of the Porcine Insulin

Precursor(PIP) gene. The authors observed a normally distributed quantity of protein expression at increasing gene dosage. The highest yield was noted at a gene dosage of 12 copies, beyond which protein expression decreased significantly. Another report describes a similar level of gene dosage (11 copies) resulting in an increase in mini-proinsulin production (Mansur *et al.* 2005b). However, these studies were done in shake flasks. It remains to be shown whether the multi-copy expression systems are capable of growth to high cell densities in large scale processes and whether the expression levels are maintained with particular consideration to the effect of oxygen transfer rates on these processes. Other investigations into insulin precursor production have focussed on the conditions of insulin precursor production. Pais-Chanfrau *et al.* (2004) showed that increasing the pH from 5.1 to 6.3 and decreasing temperature from 28$^{o}$C to 22$^{o}$C during the fed-batch phase on methanol increased mini pro-insulin production in a Mut$^{S}$ strain of *P. pastoris*. In an attempt to model the growth on glycerol and methanol in chemically defined media, Pais *et al.* (2003) grew *P. pastoris* on glycerol and methanol and determined insulin precursor production relative to growth. The authors observed a direct relationship between insulin and biomass production. However in their study, the growth rate of *P. pastoris* Mut$^{S}$ obtained on methanol and glycerol (0.093 and 0.015 /h, respectively) was much lower than that in other studies reported in the literature where growth rates on glycerol of 0.23-0.25 /h have been obtained. Additionally, the insulin yield they reported of approximately 1 g/l was lower compared to the results of Wang *et al.*(2000) who obtained 1.5 g/L with the same strain.

In a recent study, Gurramkonda *et al.* (2010) grew the Mut$^{+}$ strain of *P. pastoris* expressing two copies of the gene encoding human insulin precursor. The organism was grown in a chemically defined medium. Induction was carried out with methanol at a low concentration of 2 g/L (after it was found that methanol concentrations of 6 g/L led to uncontrollable foaming and cell lysis). Insulin precursor production was found to be approximately 3.84 g/L. Following downstream purification of the precursor, a final yield of ~99% pure insulin was recorded. A comparison of the methods used for insulin production in *P. pastoris* is presented in Table 2.2.

**Table 2. 2Summary of reported findings on insulin production in P. pastoris**

| Strain, Plasmid Copy Number | Methanol (g/L) | Induction time (h) | Final biomass (g/L) | Product concentration (g/L) | Reactor size | Growth rates (/h) | Media type | References |
|---|---|---|---|---|---|---|---|---|
| $Mut^{+/S}$, 6-8 | nd[a] | 87 | nd | 1.5 | 16L | nd | BSM | (Wang *et al.* 2000, Wang *et al.* 1999) |
| $Mut^{s}$, nd | 10 | 72 | 80-90 | 0.22 | 5L | 0.093 (gly) ; 0.011 (met) | FM22 | (Pais *et al.* 2003) |
| $Mut^{s}$, 11 | <1 | 110 | 89 | 0.3 | 2.5L | Nd | BSM | (Pais-Chanfrau *et al.* 2004) |
| $Mut^{s,}$ 11 | 1 to 0.5 | 72 | 109 | 0.25 | Shake flask (250 ml) | Nd | YP | (Mansur *et al.* 2005b) |
| $Mut^{S}$, nd | Nd | 72 | Nd | 3.6 | 5L | Nd | BSM | (Xie *et al.* 2008) |
| $Mut^{S}$, 12 | 0.8 | 96 | Nd | 0.18 | Shake flask (250 ml) | Nd | YP with methanol and glycerol | (Pais-Chanfrau *et al.* 2004) |
| $Mut^{+}$, 2 | 0.2 | 88 | 59 | 3.84 | 15L | Nd | Growth medium | (Gurramkonda *et al.* 2010) |

BSM: basal salts medium FM22: cultivation media YP: yeast-peptone

Growth medium: contains per L: glycerol, 95.2 g; potassium *di*-hydrogen phosphate, 9.4 g; yeast trace metal (YTM) solution,4.56 g; ammonium sulfate, 15.7 g; magnesium sulphate *hepta*-hydrate, 4.6 g; calcium chloride *di*-hydrate, 0.28 g; and biotin, 0.4 mg. The YTM solution contained: potassium iodide, 207.5 mg L-1; manganese sulfate, 760.6 mg L- 1; *di*-sodium molybdate, 484 mg L-1; boric acid, 46.3 mg L- 1; zinc sulfate *hepta*-hydrate, 5.032 g L-1; ferric chloride*hexa*-hydrate, 12.0 g L-1; and sulfuric acid)

## 2.13 Conclusions and research focus of this study

There is an identifiable need for greater global insulin supply especially in developing countries. To this end, yeast organisms appear best suited for heterologous human insulin production. P. pastoris in particular possesses several key characteristics including a strongly inducible promoter and high quality of recombinant protein produced functioning in a manner analogous to the human protein, that make it ideal for large-scale insulin production. Attempts to produce insulin in *P. pastoris* however, have focussed on cell-level strategies of optimising gene expression by inserting multiple copies or on iterative methods of determining parameters for process development. There is a need to develop a cultivation process that is scalable and reproducible on a reactor scale. The approach offering greatest promise in this respect is the μ-stat control method of process development described earlier in Section 2.11.3. By designing a fed-batch process that optimises biomass production on the basis of kinetic parameters derived during the batch experiments, the approach adopts a focus that addresses optimisation of biomass production while holding all other variables constant. While similar methods have been adopted in $Mut^S$ organisms for other heterologous proteins, no such approaches have been reported in the literature for the production of insulin precursor in *P. pastoris*.



## 2.14 Scope and objectives of the research

The overall goal of this research is to optimise the production of insulin by cultivation of *Pichia pastoris.* Specifically the International Centre for Genetic Engineering and Biotechnology (ICGEB) have developed a *P. pastoris* insulin producer considered for large-scale production in the developing world. Its characterisation and product development are key. It is recognised that both optimal production of biomass and subsequent expression of insulin are required. Thus the specific aims of this study are to:

i. Characterisation of the ICGEB strain across a variety of carbon sources to determine the best medium conditions for large scale manufacture

ii. Determine the growth kinetic parameters (i.e. $\mu_{max}$, $Y_{x/s}$, substrate utilisation rate) in respect of three different carbon sources: glucose, glycerol and methanol in shake flasks. From these data the best carbon source for further experiments in the bioreactor would be determined.

iii. Determine the effect of methanol concentration on growth and protease expression in *P. pastoris*

iv. Establish a fed-batch protocol to grow *P. pastoris* for insulin production based on a two-stage glycerol feed followed by methanol feeding.

v. Identify the relationship between the feeding protocol of methanol used and the recombinant product formation rate.

Certain parameters in the recombinant system, i.e. host phenotype, gene copy number, secretion signal and plasmid construct, as well as culture parameters (medium composition, pH, inoculum scale-up and temperature), while not necessarily optimal, are assumed sufficient for the purposes of large-scale process development.

The approaches taken to insulin production in the literature have often focussed on individual process parameters and the results have been widely variable. For a product of commercial interest, which requires an economically feasible process, the experimental design used as well as the approach developed must be applicable to a larger scale. Thus the experimental results obtained were used to develop a fed-batch model for an insulin production process. Further, the approach developed will be applicable to large scale process given key kinetic parameters that are experimentally determined.

# Chapter 3

# Materials and Methods

### 3.1 Introduction

This chapter presents a detailed description of the methods used in achieving the objectives outlined in the previous chapter. The maintenance of the cell culture, the preparation before each shake flask and bioreactor experiment and the subsequent analyses performed are described. The chapter presents a description of the downstream processing experiments conducted in attempting to purify, identify and quantify insulin. It concludes by providing a summary of the experimental approach.

### 3.2 Micro-organism, vector construction and transformation

The recombinant organism *Pichia pastoris* Mut$^S$ GS 115 (*his4* mutant) strain was used for all experiments. Initially designed by Invitrogen Corporation (San Diego, CA, USA), it is an auxotrophic mutant deficient in histidine dehydrogenase, thus requiring histidine supplementation. The strain was provided by the International Centre for Genetic Engineering and Biotechnology (ICGEB) at the Institute for Infectious Disease and Molecular Medicine, Medical School, UCT. The organism has a single copy of the gene encoding the human insulin precursor. The insulin gene was inserted into the pPIC9K plasmid vector and expressed under the control of the alcohol oxidase 1 (AOX 1) promoter.

### 3.3 Culture preparation

#### 3.3.1 Glycerol stocks

Stocks were prepared by inoculating three-four colonies obtained from freshly grown agar plates into 2 ml of a 85% glycerol solution contained in cryo-vials. These vials were subsequently stored at -60$^o$C. In preparing working cultures, cryovials were thawed and used to inoculate shake flasks containing 200 ml of complex medium (composition given in Section 3.4). The working cultures were prepared by streaking cells grown in shake flask cultures onto agar plates (1% yeast extract, 2% peptone, 1.5% agar prepared according to the method prescribed in the Invitrogen protocol in the presence of 1 mg/ml geneticin selective marker). These could be stored at 4$^o$C for a maximum of three months and were used to inoculate the pre-inoculum cultures.

### 3.3.2 Inoculum preparation for shake flask and bioreactor (batch and fed-batch) experiments

Pre-inocula were prepared by inoculating 3-4 colonies of *P.pastoris* from agar plates into 50 ml of yeast extract-peptone (1% yeast extract, 2% peptone, YP) broth. The flasks were incubated for 24 hours at $30^0$C on an orbital shaker at 150 rpm. By sterile transfer an aliquot of the pre-inoculum comprising 10% of the culture volume was inoculated into an inoculum flask containing YP broth. Following cultivation for 24 hours at $30^0$C cultures from the inoculum flask were used to inoculate the experimental vessel. Thus for inocula used in fed-batch bioreactor experiments, 25 ml were transferred to a separate flask containing 225 ml of YP broth, whereas for inocula used in shake flask experiments, 20 ml of pre-inoculum were transferred to 180 ml of YP broth. For batch bioreactor experiments 40 ml of pre-inoculum were added to 360 ml of YP broth containing 40 g/L glycerol.

## 3.4 Medium preparation for shake flask and bioreactor experiments

### 3.4.1 Complex medium consisting of yeast-peptone with methanol, glycerol or glucose as carbon source (YP-Met/Gly/Glu)

Complex medium was composed of yeast extract, 10 g/L, peptone 20 g/L containing either glycerol (40 g/L), glucose (39 g/L) or methanol (41 g/L) (all compounds supplied by Merck, SA and added to ensure equivalent quantities of carbon in each flask). YP medium was autoclaved within the experimental vessel (flask or bioreactor). Glucose and glycerol were autoclaved separately. Methanol was filter-sterilized using a 0.22 µm filter (Millipore) before addition. Carbon compounds were added in sterile fashion to shake flasks in the laminar-flow cabinet; addition to the reactor was via the sterilized port (described in Section 3.6). Shake flask experiments in complex medium were not buffered. The buffer used in bioreactor experiments in complex medium was 1 M potassium phosphate buffer (pH 6.0). To prepare 1 L buffer, 23 g $K_2HPO_4$ and 118.1 g $KH_2PO_4$ were dissolved in distilled water and autoclaved separately. A 100 ml of buffer was added per litre of YP medium in bioreactor experiments.

### 3.4.2 Chemically defined medium (CDM)

Chemically defined medium consisted of $(NH_4)_2SO_4$, 20 g/L; $KH_2PO_4$, 12 g/L; $MgSO_4.7H_2O$ 4.7 g/L; $CaCl_2.2H_2O$ 0.36 g/L with either 50 g/L glycerol, 50 g/L glucose or 49 g/L methanol added to shake flasks in order to ensure equivalent carbon concentrations in each flask. Glucose and glycerol were autoclaved separately and methanol was filter-sterilized (as above) before addition. For both shake flask and bioreactor experiments, a trace metals solution (4.35 ml/L) was filter-sterilized before adding to the chemically defined medium after autoclaving. The trace solution used was the PTM 1 trace solution described Cregg (1998). The medium contained (per L): 6.0 g $CuSO_4.5H_2O$, 0.08g NaI,

3.0 g $MnSO_4.H_2O$, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g $H_3BO_3$, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4.7H_2O$, 0.2 g biotin and 5 ml $H_2SO_4$, The trace salts solution (containing biotin) thus prepared is stable for up to a year.

For bioreactor experiments, the pH was maintained at pH 5.5 by addition of 5 M NaOH. Bioreactor cultivations required the addition of 1 ml silicon antifoam (Sigma Aldrich, product code A5758) at the start and as required during the process.

### 3.5 Growth curve experiments

In order to monitor growth and substrate utilisation kinetics across a variety of carbon sources and media types, shake flask and bioreactor experiments were performed. The preparation of each experiment and the growth conditions used are described below. The maintenance of aseptic conditions in order to maintain a mono-culture in all the experiments was monitored by routine observation of the cell culture under the microscope. In order to ensure reproducibility, the experiments described below were performed in triplicate unless stated otherwise in Chapter 4.

#### 3.5.1 Shake flask experiments

Shake flask experiments were conducted in chemically defined and complex medium containing glucose, glycerol or methanol. The media composition and preparation is described above. The experiments were conducted in 1 L flasks containing 200 ml working volume. After autoclaving the medium and sterile addition of the carbon source, the inocula for the experiments were added. The inoculum preparation is described in Section 3.2.2. A 10% v/v inoculum was added to each flask. A sample was taken at the start of the experiment in order to determine the initial biomass and substrate concentrations and pH. Subsequently, the flasks were incubated on an orbital shaking platform at a gyratory speed of 150 rpm in an incubator set to 30$^{o}$C. Oxygen transfer to the flask was via the sterilized cotton wool bung sealing the flask. Samples were obtained every 2 hours (unless otherwise stated) by sterile transfer of 2 ml of the culture broth to an Eppendorf tube. The biomass and cell concentration were monitored as subsequently described in Section 3.8. The supernatant was refrigerated for subsequent substrate analysis of glucose, glycerol, ethanol or methanol concentrations.

#### 3.5.2 Shake flask experiments to investigate growth on methanol

Three separate shake flask studies were conducted to investigate the effect of methanol on *P. pastoris.* In the first methanol experiment, the influence of methanol on protease formation was considered. The experimental set-up was identical to shake flask experiments described in Section

3.5.1. Cells were grown on YP-gly (40 g/L) for 48 hours before addition of filter-sterilised methanol at 1, 5, 7.5, 10, 20, 30 and 40 g/L. A control flask identically composed but without methanol addition was used. Thereafter, flasks were incubated for a further 24 hours, at the end of which samples harvested from each of the flasks were analysed for their protease activity (see Section 3.8.4 for protease analysis protocol).

In the second experiment, the effect of methanol on cells grown on glycerol prior to methanol addition was considered. Cells were initially grown in chemically defined medium containing glycerol (40 g/L). After 24 hours of growth, methanol was added at 0, 5, 7.5, 10, 20, 30 and 40 g/L. Biomass and cell concentration were monitored as described in Section 3.9. Substrate analysis was by HPLC and the protease assay was conducted to determine the levels of extracellular protease activity.

In the third experiment, the ability of methanol to support growth in the absence of additional carbon source was considered. Cells were inoculated from a yeast-extract peptone inoculum into a 200 ml volume of chemically defined medium with no additional carbon source other than methanol. To the experimental flasks 0, 5, 7.5, 10, 20, 30 and 40 g/L methanol were added at inoculation. Growth and substrate utilisation were monitored over 24 hours. Protease release was monitored at the final time point (24 hours). Thus the effect of methanol on growth (without prior glycerol addition) was investigated.

### 3.6 Reactor set-up and bioreactor experiments

Bioreactor experiments were conducted in the New Brunswick Scientific BIOFLO 110 series bioreactor system (USA). The reactor vessel had a total internal volume of 7 L with a maximum working volume of 5 L. It was configured for online monitoring of pH, temperature and dissolved oxygen to ensure the maintenance of optimal growth conditions. The pH of the medium was measured using a pressurised pH probe (Mettler Toledo). The pre-set pH value of pH 5.5 was maintained by automatic titration of 5 M NaOH for the chemically defined medium, whereas no pH control was necessary for the complex media cultivations due to addition of a phosphate buffer (composition given in Section 3.3) at the start of the experiment. Dissolved oxygen was measured with a polographic dissolved oxygen probe (Mettler Toledo) fitted with a Teflon membrane. The dissolved oxygen probe was polarised for 4-6 hours after autoclaving the reactor containing medium before calibration. Temperature was monitored using a thermoprobe fitted into the reactor. The preset temperature ($30^{o}C$) was maintained by means of a heating jacket and water circulation through the internal cooling coil. Inlet air and supplemented oxygen were filtered through an inlet

air filter (Millipore Aervent-50) and supplied through a sparger immediately beneath the lowest impeller. Adequate mixing of the reactor contents was ensured by the presence of two six-blade Rushton impellers and four baffles. The reactor vessel was sterilized at 121$^{o}$C for 20 minutes. During operation, evaporative losses were reduced by cold water flow through the combined air exhaust condenser. An air filter (Millipore Aervent 50) attached to the exhaust ensured the release of sterile gas into the surrounding environment. The reactor set-up is shown in Figure 3.1.

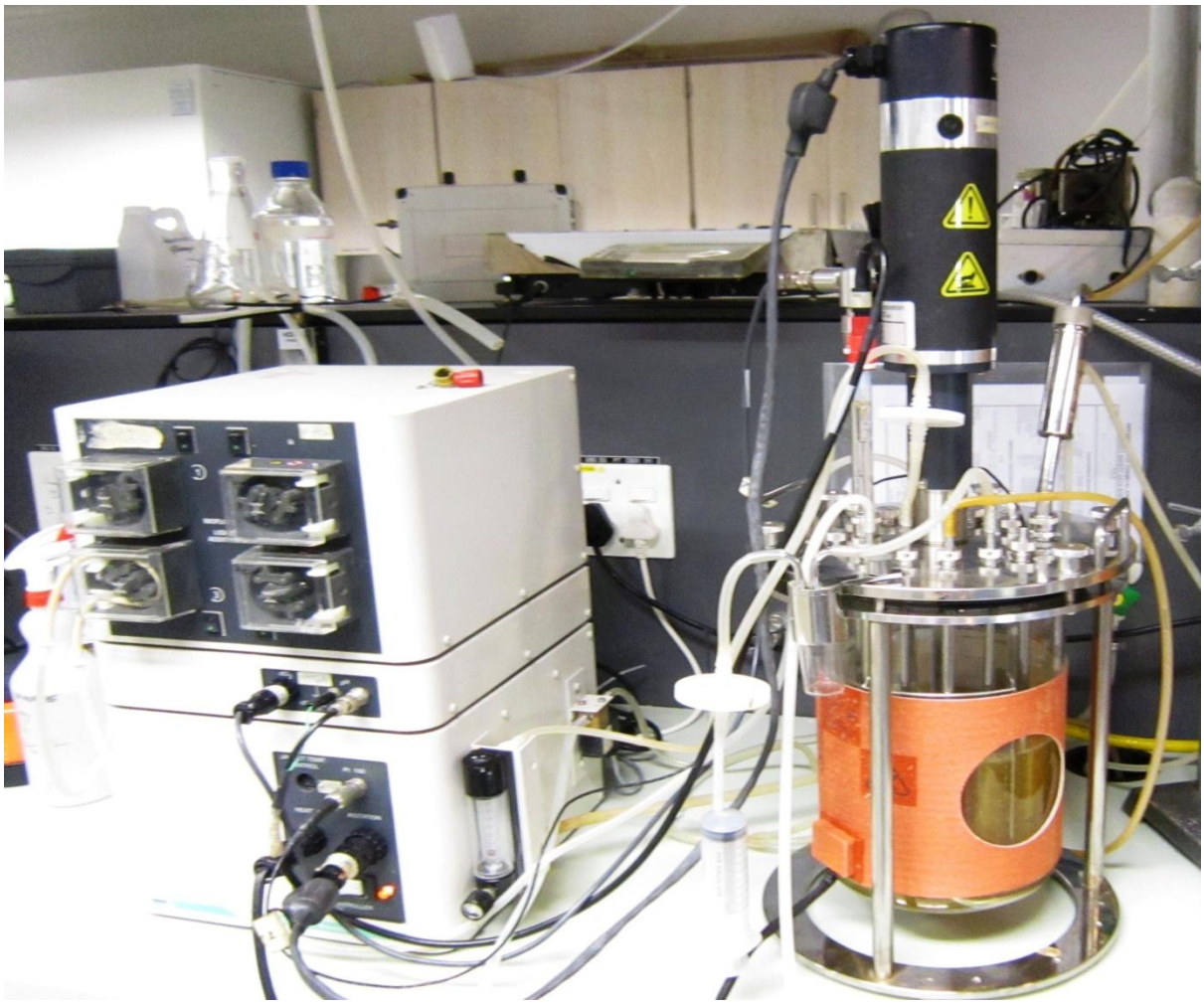

**Figure 3. 1 Bioreactor setup for growth on YP-Glycerol medium** after configuration of initial temperature, pH and dissolved oxygen concentration.

The working volume for each batch bioreactor experiment was 4 L. Hence, 3.4 L of yeast-peptone or chemically defined medium (as described in Section 3.3.1) was prepared in the reactor before autoclaving. Pure glycerol was weighed out (160 g) and autoclaved separately, resulting in 40 g/L final glycerol concentration for cells grown in complex medium and 50 g/L for cells grown in chemically defined medium. A 400 ml volume of yeast peptone medium containing inoculum was added at the start of the experiment. Glycerol, inocula, trace salts (for bioreactor) and antifoam

were added to the reactor at the start of the experiment. To ensure sterile inoculation, all contents of the inocula (culture, glycerol, antifoam, buffer solution) were added to an autoclaved flask with a connection valve. The valve was subsequently connected to an entry portal on the vessel and the flask drained into the reactor by gravity. Samples were removed from the reactor via the sampling port in 10 ml volumes in sample bottles. From this volume of sample, analyses were performed to determine cell concentration, gravimetric cell dry weight and substrate concentration.

The *P. pastoris* bioreactor was operated at $30^0$C. The air flow rate was set at 1.2L air/L culture-minute. Agitation was set at between 600-1000 rpm to maintain dissolved oxygen concentration greater than 20% of saturated oxygen concentration on sparging with air. If the oxygen uptake rate exceeded the oxygen transfer rate even at 800 rpm, oxygen was added as required by mixing pure oxygen with air to increase the oxygen transfer rate. For growth in chemically defined medium, the pH was maintained at pH 5.5 by addition of 5 M NaOH. Medium was prepared as described in Section 3.3.1. Samples were taken at regular intervals (approximately every 2 hours) via the sampling port for optical density (OD) measurements, substrate and protein analysis. Analyses performed are described in detail in Section 3.8.

### 3.7 Fed-batch experimental set-up

For the initial batch phase of the experiment the bioreactor was set up as described in Sections 3.2 and 3.6. The reactor was filled with 2.5-3 L of chemically defined medium with glycerol (50 g/L) as the sole carbon source. Cells were grown for approximately 24 hours in batch phase allowing for complete glycerol exhaustion. Upon glycerol depletion, as measured using HPLC, a fed-batch phase was initiated with glycerol as the sole carbon source. Sterilized glycerol (200 g/L) in chemically defined medium was fed at an exponential, predetermined growth rate. The feed regime was calculated according to Equation 3.1

$$F = \frac{\mu XV}{Y_{X/S}} e^{\mu(t)} \qquad \text{Equation 3.1}$$

where μ was the predetermined growth rate, X the cell concentration at the end of the batch phase, V the reactor volume at the start of the fed-batch phase, $Y_{x/s}$ the yield of biomass upon substrate at the end of the batch phase, $S_0$ the initial substrate feed (200g/L), *t* the duration of the fed-batch phase. The fed-batch system was operated at quasi-steady state, with the nutrient consumption rate being approximately equal to the nutrient feed rate and the residual substrate being close to zero (S≈0). The calibrated pump was configured to feed glycerol at the predetermined flow rate. By constant monitoring the feed bottle gravimetrically during fed-batch operation, the flow rate could

be calculated using solution density and adjusted to deliver the desired rate. Additionally, the substrate concentration within the reactor was constantly monitored by HPLC to ascertain no accumulation of excess glycerol and to determine whether ethanol or acetate (known repressors of the AOX promoter) was produced.

During fed-batch operation the dissolved oxygen was maintained at 20-30% of air saturation by increasing the agitation speed up to a maximum of 800 rpmor by augmenting the air supply with pure oxygen. The reactor volume was allowed to increase from the batch culture volume of approximately 2.5 L to a maximum of 4L. At this point, the glycerol feed was stopped and the methanol feed was initiated. Initially a small quantity of methanol at 5 g/L was added to acclimatize the cells to methanol before fed-batch addition of methanol. Thus 20 g of pure methanol was filter sterilized into an autoclaved vessel before addition to the reactor under sterile conditions. After 2 hours (or when the reading of the dissolved oxygen concentration increased), chemically defined medium containing 200g/L methanol was added in fed-batch fashion to the reactor. The feed rate was iteratively determined by measuring the rate of depletion of methanol. Methanol was subsequently fed at this rate after adjusting for the evaporation rate (0.488g/Lh). Samples were withdrawn periodically to measure residual methanol concentrations, cell concentration and protein concentration. Fed-batch cultivation was continued until the maximum working volume of 5 L was reached.



### 3.8 Analytical Methods

#### 3.8.1 Cell concentration by optical density measurement

Cell concentration was measured by monitoring the light scattering effect of *P. pastoris* cultures in a spectrophotometer at a wavelength of 600 nm. In order to maintain the linear relationship between cell concentration and absorbance, samples were diluted to maintain an absorbance reading of 0.700 or below in cuvette of height 45 mm and width 12.5 mm

#### 3.8.2 Biomass measurement

Biomass was quantified gravimetrically in terms of the dry cell weight produced over time. Two ml of cell suspension was added to dried, pre-weighed Eppendorf tube and centrifuged at 4400 rpm for 5 minutes. The supernatant was retained in a separate Eppendorf tube for substrate analysis. The pellet was dried in an oven at 80$^{o}$C overnight and stored in a desiccation jar. Cell dry weight was measured until a constant biomass value was achieved. In order to infer cell dry weight measurements from optical density measurements, the experimentally determined cell dry weight

measurements were correlated with the optical density measurements obtained. A linear relationship of correlation coefficient ($R^2$) of 0.936 was observed (shown in Appendix B); hence the biomass at any time point could be determined from the OD.

### 3.8.3 Glucose, glycerol, methanol and ethanol analysis

Glucose, glycerol, methanol and ethanol concentrations were quantified by High Pressure Liquid Chromatography (HPLC). The HPLC system consists of a Waters 520 HPLC pump and Waters 410 Differential Refractometer (Waters Millipore, Milford, MA, USA). The HPLC column used was a Biorad Aminex HPX-87H Column 125-0140, 300 x 7.8 mm (Anion High Resolution) pre-packed HPLC carbohydrate analysis column, hydrogen form, 9 µm particle size, 8% cross linkage, operating within a pH range of 1–3. Attached to it was a guard column to remove any proteins and cellular materials to minimize fouling of the column. The system was run isocratically using a mobile phase of 0.01 M $H_2SO_4$ at a flow rate of 0.6 ml/min. The pressure in the column did not exceed 2000 psi. Sample injection volumes of 20 µL were used. Breeze detection software was used to compute the peak areas of standard solutions of each compound. To quantify the concentrations of each carbon compound, standard solutions of each compound were prepared at increasing concentrations (in distilled water). The resulting standard curves are shown in Appendix C.

### 3.8.4 Protease analysis

The protocol used was provided by suppliers of the Universal Protease Substrate. The substrate, casein-resorufin-contains casein labelled with N-(resorufin-4-carbonyl)-piperidine-4-carbonic acid supplied by Roche Applied Science (catalogue number 11734334001)according to the modifications of Basson (1997).The following reagents were prepared in distilled, de-ionized water: i) Incubation buffer consisted of Tris-HCl buffer (0.2 mol/L Tris–HCl, pH 7.8, 0.02 mol/L $CaCl_2$); ii) Stop reagent was made up of trichloroacetic acid (5% w/v); iii) Assay buffer: Tris-HCl (0.5 mol/L, pH 8.8); iv) Substrate solution: Casein, resorufin-labelled (0.4% w/v). Aliquots of 50 µL were transferred to Eppendorf tubes and frozen at -20$^{o}$C until required.

Yeast culture supernatants were thawed and re-centrifuged for five minutes to remove any remaining yeast cells. The two ml volume of supernatant was transferred to new Eppendorf tubes. A 50 µL volume of incubation buffer was added to Eppendorf tubes containing 50 µL of casein substrate. A 100 µL volume sample of the yeast supernatant was added for analysis. For the negative control (and in order to 'blank' the spectrophotometer), 100 µL of deionised distilled water was added.

The contents of the tube were mixed gently and the tubes were incubated overnight at 37°C. To halt protease activity, 480 µL of 'stop' buffer were added. The tubes were incubated for 10 minutes before centrifuging for five minutes. A 400 µL aliquot of supernatant was pippetted into a 1 mL cuvette and 600 µL of assay buffer added. The absorbance readings were measured at 574 nm.

The assay was calibrated by constructing a calibration curve using proteinase K (supplied by Roche) diluted to produce solutions of defined activity (the curve is shown in Appendix F). The samples were incubated with casein resorufin as described above. However, the incubation time was reduced to 15 minutes due to rapid degradation of casein-resorufin by pure protease. Proteinase K has 2.5 Units/mg activity according to the manufacturer's instructions. One unit of enzyme activity is defined as that amount of enzyme that liberates Folin-positive amino acids and peptides corresponding to 1 µmol tyrosine in 1 minute at 37°C using denatured haemoglobin as substrate.

### 3.8.5 Protein quantification

Total protein in the samples was assayed using the Pierce Bicinchoninic acid (BCA) Total Protein Analysis Kit (Pierce Chemical, Rockford, IL, USA). Five µL of protein samples were incubated with 200µL of the BCA reagent at 37°C for 30 minutes before analyzing the absorbance on a microtitre plate reader. In order to quantify the total level of protein, the absorbance measurement obtained at a wavelength of 562 nm was used to determine the concentration of the protein from the calibration curve. The calibration curve was obtained by plotting the absorbance readings of known bovine serum albumin (BSA) protein concentrations against the concentrations of each BSA protein standard.

## 3.9 Approaches to detection of insulin precursor

### 3.9.1 Overview

The strain of *P. pastoris* used was designed to secrete the insulin precursor to the extracellular matrix. The first purification step required solid-liquid separation to remove the biomass. The supernatant containing the histidine-tagged insulin precursor was subsequently run through an affinity purification column in which the histidine tag was used to separate insulin precursor from other proteins present in the extracellular matrix. The protein fractions harvested from the various affinity purification steps were analyzed on a one-dimensional gel to determine which fraction most likely contained insulin precursor. This was determined by identifying the fraction that contained the 15 kDa size fragment corresponding to insulin precursor. The sample containing this fraction was subsequently run on the HPLC column. This approach however (as will subsequently be shown) was unsuccessful in identifying or quantifying the level of insulin precursor present. In order to identify

whether insulin was being secreted, antibody detection via Western Blot and mass spectrometry methods were used. The various methods by which insulin precursor identification, purification and quantification were attempted are described below.

### 3.9.2 Affinity purification to purify histidine-tagged insulin precursor

The supernatant harvested during sampling was probed for insulin. A commercial affinity purification kit was used for the purification of insulin based on its attached histidine tag. The Protino Ni-TED 2000 kit (Macherey and Nagel,USA) consists of affinity columns containing a special silica resin that allows selective binding of the histidine tagged protein. Lysis elution wash buffer (LEW) buffer and the elution buffer were used. The working solutions and purification protocol followed were as specified by the manufacturer, with recipes provided in Appendix I.

The column was equilibrated using 4 ml LEW buffer. A two ml volume of supernatant containing the putative insulin precursor was loaded onto the column. This was washed twice with 2 ml of LEW buffer. Each fraction was collected in a separate tube. Subsequent elution steps involved washing the column with three volumes of elution buffer collecting each eluted fraction separately to ensure that all the histidine tagged protein was harvested. Protein constituents in each fraction were separated by one-dimensional gel via electrophoresis to determine the fractions containing a high concentration of the insulin precursor. These fractions were subsequently analysed via RP-HPLC (details given in Section 3.9.4).

### 3.9.3 Sodium dodecyl sulphate polyacrylamide- gel electrophoresis (SDS-PAGE)

In order to identify insulin precursor, the fractions collected following affinity purification were run on a one-dimensional electrophoretic gel. A 15% SDS-Polyacrylamide gel was cast using the tris/tricene buffer system of Schaegger and von Jagow (1987). Details are given in Appendix G. Gels were run for one hour at 100 V using the Mini-Protean gel casting system (Biorad Labs, Richmond, CA, USA). Gels were stained using Coomassie blue (0.25%) and destained using de-stain solution (10% acetic acid, 20% ethanol in 1 L distilled water). The fractions thought to contain the putative insulin precursor were run on the HPLC column as described below.

### 3.9.4 Insulin precursor identification via HPLC

Samples potentially containing the insulin precursor were run through a RPC (Reverse Phase Column) Resource (Amersham Pharmacia Biotech, 17-1182-01) column. The two mobile phases used during the gradient elution, A and B, were made up as follows:

Mobile phase A consisted of 210 ml of sulphate buffer solution (132.1 g $(NH_4)_2SO_4$; 14 ml $H_2SO_4$ ; pH 2) and 790 ml of distilled water per L. Mobile phase B consisted of 105 ml of sulphate buffer solution and 40% acetonitrile made up to one litre with distilled water. The gradient elution protocol is shown in Table 3.1.

**Table 3. 1 Gradient elution protocol for identification of insulin precursor via Reverse Phase HPLC**

| Gradient | Duration | Volume | Flow rate | Column Volumes (CV) |
|---|---|---|---|---|
| 0-45% B | 10 min | 50 ml | 4.5%/min | 16.6CV |
| 45% B | 10 min | 50 ml | | 16.6CV |
| 45-75% B | 10 min | 50 ml | 4.5%/min | 16.6CV |
| 75% B | 1.6 Min | 8 ml | | 2.7CV |
| 75-100% B | 3 min | 15 ml | 8.3%/min | 3 CV |

The sample injection volume was 20 µL and the mobile phase flow rate used was 3 ml/min. Due to the lack of insulin standards, commercially available insulin was used to detect the insulin peak. Standards were prepared from commercially available Insulin (Lantus and Apidra, Sanofi-Aventis). The peaks indicating insulin were determined by running samples containing the purified form of each component in the insulin preparation. Each milliliter contained the following components: glycerol (85%v/v concentration), polysorbate 20 (10 mL vial only), *m*-cresol, zinc and water for injection. Each milliliter of APIDRA contained100 units of insulin glulisine. It also contained excipients: *m*-cresol, trometamol, sodium chloride, polysorbate 20 and water for injection. APIDRA has a pH of approximately 7.3 and is adjusted by addition of aqueous solutions of hydrochloric acid and sodium hydroxide (Sanofi Aventis, 2011).

### 3.9.5 Western blot detection of insulin precursor

To confirm whether the HPLC peaks produced by samples obtained from the reactor were due to insulin precursor, Western blot experiments were performed. The recipes for the various solutions used during this phase of the experiment are given in Appendix I. The cultivation samples were centrifuged and the supernatant from induced and non-induced cells removed for western blotting. The cells in the pellet were re-suspended in 0.05 M Tris/0.01 M EDTA buffer containing COMPLEAT protease inhibitor (Roche, USA). The cell suspension was subsequently lysed by ultra-sonication until few intact cells (<100) were visible under the microscope at 100 X magnification (generally less than a percent of initial intact cells by crude estimation). The cell lysate was clarified by centrifugation to

removed debris and cells. Protein in the supernatant samples was present at very low concentrations (<0.04 µg/µl). Thus, to increase the protein concentrations, samples were treated with trichloroacetic acid and acetone according to a protocol by Luis Sanchez of Caltech (2001). To 1 ml of sample, 250 µL of trichloroacetic acid (TCA) was added to 1 ml of sample. The sample was incubated for 10 minutes at $4^0$C, followed by microcentrifugation for 5 minutes. The supernatant was removed, leaving the white protein pellet intact. This pellet was washed with 200 µL of ice-cold acetone and spun in a microfuge for 5 minutes. The pellet was dried at $90^0$C on a heating block to drive off the acetone. For SDS-PAGE, 2X sample buffer (composition: 126 mM Tris-HCl; 20 % v/v glycerol; 4% v/v SDS; 0.02 % v/v bromophenol blue at pH 6.8 ± 0.02)was added to the supernatant and cell lysate samples before boiling them at $95^0$C and loading them onto the polyacrylamide gel.

The proteins in the samples were separated according to size and charge on a 5 % stacking gel and a 15 % resolving gel (recipe given in Appendix I). The gels were electro-blotted onto polyvinylidene flouride (PVDF) membranes as specified by Harlow and Lane (1988). Gel transfer occurred for 1 hour at 120V.The membranes were blocked in a 5% skim milk buffer for an hour at room temperature. The membranes were subsequently washed with a Tris/Tween 20 buffer, incubated overnight at $4^0$C with mouse anti-proinsulin antibody (Sigma-Aldrich, SA) diluted 1 in 4000 in milk buffer. Following this, the membranes were rewashed with tris/tween20 buffer to remove non-specifically bound protein and incubated for 1 hour in rabbit anti-mouse IgG antibody-horseradish peroxide conjugate in a 1 in 1000 dilution in 5% milk buffer (Pierce Chemical, Rockford, IL, USA). After a wash cycle with tris/tween 20 buffer, membranes were reacted with NEN Renaissance Enhanced Western Blot Chemiluminescence reagent (Pierece Chemicals), blotted dry and exposed to Kodak X-Ornat Blue (Kodak, Rochester, NY, USA) X-ray film.

### 3.10 Matrix-Assisted laser desorption/ionization analysis (MALDI)

#### 3.10.1 Overview

To prepare samples for MALDI, it was important to concentrate the protein present in the extracellular matrix. This was done by freeze-drying (lyophilisation). The concentrated protein was re-suspended in distilled water before being precipitated by ammonium sulphate and acetone addition. The protein thus obtained was run on a one-dimensional polyacrylamide gel and visualized using Coomassie blue dye. Following destaining of the gel, the putative insulin precursor containing bands were excised and processed via MALDI mass spectrometry.

### 3.10.2 Lyophilisation to concentrate extracellular proteins

Culture medium was clarified to remove suspended cells by centrifugation in a Beckman-Rotor centrifuge using a JA 10 rotor at 10 000 rpm for 20 minutes at 4$^{o}$C. A supernatant volume of 50 ml was lyophilized overnight to obtain a fine powder containing precipitated salts and extracellular proteins from the extracellular matrix. The powder was re-suspended in 20 ml of distilled water before further processing for MALDI analysis.

### 3.10.3 Intra-cellular protein extraction using ultra-sonication

Cell walls were disrupted using sonication to obtain the total intracellular protein. A two ml volume of cell suspension was placed on ice and sonicated at 7 µm of wave amplitude at times ranging from 5-30 minutes using a MSE Soniprep 150. Samples were periodically examined under the microscope to determine the extent of cell disruption due to sonication. Upon complete disruption, the lysed suspensions were transferred to Eppendorf tubes and clarified via centrifugation to remove cellular debris. The clarified lysates were stored separately at 4$^{o}$C for subsequent ammonium sulphate precipitation.

### 3.10.4 Protein precipitation from lysed cell suspensions and extracellular matrix

Intracellular proteins were precipitated via ammonium sulphate precipitation. Ammonium sulphate was continuously dissolved in a solution containing cell lysate until the proteins present were precipitated out. The final concentration of ammonium thus added was 52 g/100 ml. The precipitate was dissolved in water and ammonium sulphate removed by buffer exchange using 3 kDa NMW cut-off filters (Millipore). The extracellular protein suspension obtained by lyophilisation (Section 3.10.2)was precipitated via addition of acetone. To a 2 ml volume of sample, 4 volumes (i.e. 8 ml) of ice-cold acetone were added. These were removed by dissolving acetone in a miniscule quantity of distilled water before evaporation using a roto-evaporator. The process was repeated five times before the sample solvent was removed. The protein was further concentrated by ultrafiltration by passing samples through a 3kDa NMW cut-off filter (Millipore).

The samples obtained were subsequently quantified via bicichoninic acid(BCA) assay (described in Section 3.9.5) and run on a 15% SDS-PAGE gel. Protein bands were visualized using Coomassie blue dye. The dye was added to the gel and incubated overnight on an orbital shaker before being destained using destain solution (10% acetic acid, 40% ethanol made up in 1L of distilled water). The gel containing the putative insulin precursor band was sent to the MALDI facility at the Centre for Proteomics and Genetics Research (UCT) where the analysis described below was conducted.

### 3.10.5 MALDI sample analysis

#### *3.10.5.1 Digestion*

All reagents used were analytical grade or equivalent. Gel slices supplied were de-stained in a1.5 ml Eppendorf tube with a 50:50 solution of 200 mM $NH_4HCO_3$: Acetonitrile (Burdick & Jackson; Sigma) until clear. Samples were dehydrated and desiccated before reduction with 2 mM triscarboxyethyl phosphine (TCEP; Fluka) in 25 mM $NH_4HCO_3$for 15 minutes at room temperature with agitation. Excess TCEP were removed and the gel pieces again dehydrated. Cystine residues were carbamidomethylated with 20 mM iodoacetamide (Sigma) in 25 mM $NH_4HCO_3$ for 30 minutes at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 25 mM $NH_4HCO_3$ followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in trypsin (Promega) solution (20ng/uL) and incubating at 37 °C overnight. Peptides were extracted from the gel pieces once with once with 50µL0.1% trifluoroacetic acid (TFA) (Sigma). The samples were dried down and 200µL water added and concentrated to less than 20µL to remove residual $NH_4HCO_3$.

The samples were dried and re-dissolved in 0.1% TFA. The samples were mixed with α-cyano-4-hydroxy-cinnamic acid (CHCA) to give a final concentration of 5 mg/mL CHCA. The sample-matrix mixture was spotted manually onto a MALDI target plate.

#### *3.10.5.2 Mass spectrometry*

MALDI MS was performed using a 4800 MALDI ToF/ToF system (AB SCIEX) with instrument control through a 4000 Series Explorer. Parent spectra were acquired in reflector positive mode at a laser intensity of 3500 arbitrary units using 600 laser shots per spectrum. The grid voltage was set to 16kV. Spectra were internally calibrated using trypsin autolytic fragments. Fragmentation data was acquired in positive mode with a deceleration voltage of 1kV. The spectra were acquired with a laser intensity of 4500 arbitrary units and 1600 shots per spectrum.

#### *3.10.5.3 Data Analysis*

Database interrogation was performed with the Mascot algorithm using the MSDB database on a GPS workstation. Search parameters were as follows:

Species – all entries;

Enzyme – trypsin;

Maximum number of missed cleavages -1;

Fixed modifications – carbamidomethyl (C);

Variable modifications oxidation (M); deamination (N/Q); pyroglutamic acid

Precursor tolerance - 100 ppm.

Fragment tolerance – 0.2 da

### 3.11 Approach to experimental investigation

To meet the objectives set out in Section 2.14 the following experiments were performed:

- Batch studies in shake flasks to characterise the growth of *P. pastoris* in chemically defined and complex media containing glucose, glycerol and methanol.
- Bioreactor studies to establish the kinetic parameters on optimal carbon source established in the above experiments. The parameters $Y_{X/S}$ and $\mu_{max}$ were used to determine the fed batch experimental strategy to optimise biomass production
- Determination of the effect of methanol on growth by cultivating *P. pastoris* at 0, 5, 7.5, 10, 20, 30 and 40 g/L methanol in chemically defined and complex medium. The effect of pre-culturing cells on glycerol before addition of methanol was additionally considered.
- Investigation of the effect of methanol on protease release by similarly incubating cells in complex medium containing methanol at various concentrations.
- Fed-batch studies where cells were grown in batch in chemically defined medium containing 50 g/L glycerol. Upon glycerol exhaustion, medium containing glycerol was fed to the reactor to maintain a predetermined exponential growth rate, in order to boost biomass production prior to induction of the AOX promoter by methanol addition
- Insulin precursor identification was attempted by RP-HPLC after affinity purification, Western blot detection, and Matrix Assisted Laser Desorption Ionisation (MALDI)

# Chapter 4

# Results and discussion

### 4.1 Introduction

The overall aim of this project was to design a scalable fed-batch insulin production process. Results from the following experimental investigations are presented:

- Selection of media and optimal carbon source through shake flask studies. Cells were grown in complex and chemically defined media containing equivalent carbon concentrations (~50 g/L in chemically defined and ~ 40 g/L in complex media) of glucose, glycerol and methanol. The carbon source chosen from these experiments was used for larger scale bioreactor studies.

- Kinetic parameters ($Y_{X/S}$, $\mu_{max}$ and dS/dt) were compared in bioreactor experiments on cells grown in complex and chemically defined media containing 40 and 50 g/L glycerol, respectively. These kinetic parameters were used to design fed-batch experiments.

- Three separate shake flask experiments were performed to investigate the effect of methanol on the growth of :*P. pastoris*

    - Protease expression was investigated at residual methanol concentrations of 0, 5, 7.5, 10, 15, 20, 30 and 40 g/L in cells grown in complex medium.

    - The effect of methanol addition at residual methanol concentrations of 0, 5, 10, 15, 20, 30 and 40 g/L to cells grown on glycerol (50 g/L)

    - The effect of methanol addition alone (without prior glycerol addition) was determined for the same range of concentrations used above.

- Two preliminary fed-batch experiments based on the kinetic parameters to optimise the fed-batch process were followed by three optimised fed-batch experiments with duplicate experiments at a chosen pre-determined growth rate of 0.17 /h and a single experiment at a higher pre-determined growth rate of 0.21 /h.

- RP-HPLC, Western Blot Detection and MALDI analysis were used to determine the presence of insulin precursor.

## 4.2 Optimising biomass production in shake flask experiments

### 4.2.1 Growth of *P. pastoris* in chemically defined media containing various carbon sources in shake flasks

Growth was studied in chemically defined medium containing 49 g/L glucose, 50 g/L glycerol and 52 g/L methanol, each equated to a molar concentration of 0.54 M glycerol.

Figure 4.1 shows data obtained from triplicate sets of shake flask experiments investigating growth of *P. pastoris* in chemically defined medium containing glycerol. Exponential growth commenced immediately upon inoculation with no significant lag phase due to the rapidly dividing cells present in the inocula. A maximum specific growth rate of 0.09 ± 0.007 /h was obtained from the slope of the natural logarithm of the biomass concentration as a function of time. Glycerol concentration decreased from 55 ± 4 g/L at t = 0 h to 15 ± 4 g/L after 12 hours of growth, an approximately 72% depletion of the initial glycerol charge. The HPLC analytes observed showed no ethanol or acetate production. Cells reached a maximum biomass concentration of 5.4 ± 0.3 g/L at stationary phase after 24 hours of growth. At the end of the experiment, after 36 hours of growth, the residual glycerol concentration was 5 ± 0.4 g/L leading to the onset of stationary phase. Cells entered stationary phase despite the presence of residual glycerol suggesting an alternative nutrient was limiting growth. There was a high degree of reproducibility in the growth and substrate utilisation profiles observed, as well as in maximum cell concentrations achieved. It should be noted that pH was not monitored during the execution of this experiment and thus its effect on growth cannot be ruled out. However, according to the literature, *P. pastoris* growth occurs over a broad range of pH conditions, capable of tolerating a pH range from 5.3 to 8.7 (Chang *et al*. 2006, Cos *et al*. 2006. Thus the effect of pH on growth is most likely insignificant.

Figure 4.2 shows shake flask growth of *P. pastoris* in chemically defined medium (identically constituted as above) containing 41 ± 2 g/L glucose measured at the start of the experiment. Similar to growth in Figure 4.1, cells grown on glucose did not experience a significant initial lag phase before achieving the maximum specific growth rate ($\mu_{max}$) of 0.158 ± 0.002 /h within the first 12 hours of cultivation.

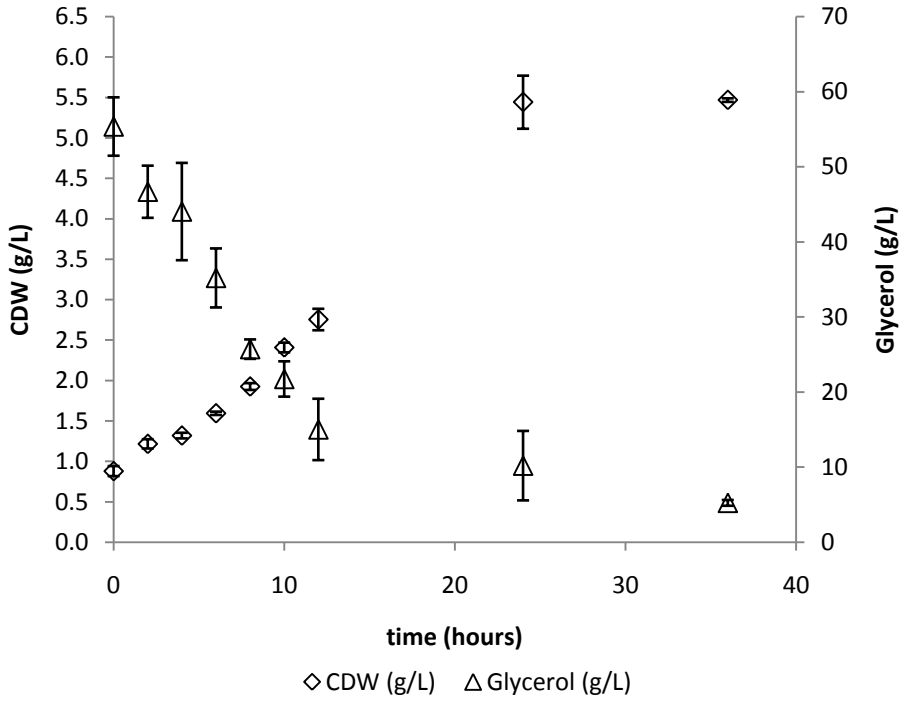


**Figure 4. 1 Growth profile of *P. pastoris* in chemically defined medium containing 55 ± 4 g/L glycerol.**
Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) determined via correlation with $OD_{600\ nm}$ (◊) and glycerol utilisation (g/L) (Δ) are shown. Results shown are representative of three independent experiments and error bars are included.

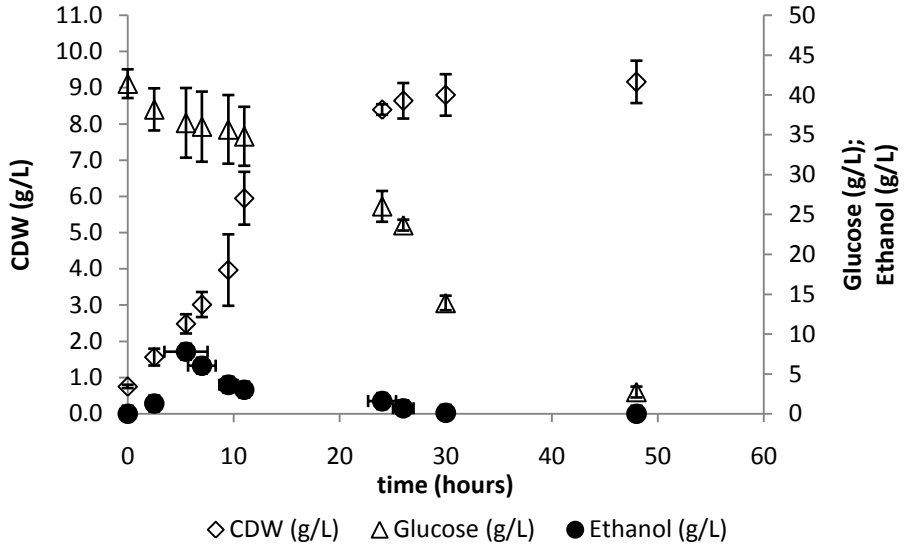


**Figure 4. 2Growth profile of *P .pastoris* in chemically defined medium containing 41 ± 2 g/L glucose.**
Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) determined via correlation with $OD_{600\ nm}$ (◊); glucose utilisation (g/L) (Δ) and ethanol production (•) as shown. Results shown are representative of three independent experiments and error bars are included.

The cells approached stationary phase after 24 hours of growth. Following the initial exponential phase, the average specific growth rate (μ) slowed over the next 12 hours, μ for 12-24 hours being 0.032 ± 0.006 /h, suggesting possible oxygen or nutrient limitation. The effect of changes in pH,

although not determined at the end of the experiment, could also have contributed to the slowing of growth, however it has been noted that P.pastoris tolerates a broad range of pH.

Final biomass concentration at the end of 30 hours growth was 8.8 ± 0.6 g/L. The consumption of glucose began upon inoculation of *P. pastoris*. Glucose concentration dropped from 41 ± 2 g/L at inoculation (t = 0 h) to 36 ± 4 g/L after 5 hours of growth with a concomitant increase in biomass from 0.8 ± 0.04 g/L to 2.5 ± 0.3 g/L. During this period, ethanol was also produced. Ethanol reached its highest level of 8 ± 2 g/L at the end of 5.5 hours of growth. From the eighth hour of growth to the final sample point at the $48^{th}$ hour, the ethanol concentration decreased to zero as biomass increased to 9.2 ± 0.6 g/L and glucose concentration decreased to 2.7 ± 0.8 g/L. Across each of the flasks, this general trend was consistently observed, suggesting that in all instances ethanol was fully utilised towards the end of growth in shake flasks. It appeared that upon inoculation, cells used glucose available to produce biomass and ethanol during the first 5.5 hours of growth. Subsequently, cells utilised the ethanol and glucose available in the medium for biomass production resulting in a depletion of these carbon compounds. Figure 4.3 shows the growth of *P. pastoris* in chemically defined medium containing methanol. The initial methanol concentration was 45 ± 2 g/L. The rate of methanol consumption was slow over the 30 hour growth period observed. In the fastest period of growth, i.e. during the first 12 hours, the growth rate was 0.035 ± 0.004 /h, the rate of methanol depletion was 0.78 ± 0.11 g/Lh. When the rate of methanol evaporation was accounted for (as determined in Section 4.5.5), the methanol depletion attributable to biomass formation was 0.468 ± 0.005 g/L.h during the first 12 hours of growth. The substrate utilisation rate over 24 hours, accounting for methanol evaporation, was 0.148 ± 0.02 g/L.h. The average biomass yield obtained during growth was 0.17 ± 0.005 g/g. The highest biomass concentration achieved was 1.8 ± 0.3 g/L. No ethanol or acetate production was observed upon growth on methanol in chemically defined medium. Shake flasks were inoculated with a 10 % inoculum as previously described (in Section 3.3). The inoculum contained yeast extract and peptone. Figure 4.4 shows growth in chemically defined medium with no added carbon. The cells grew at 0.08 /h, producing a small increase of biomass (1.393 g/L).

The kinetic parameters computed for growth in chemically defined medium with various carbon sources added as well as a control flask in which no carbon was added are shown in Table 4.1. The results allowed for some important comparisons between the carbon sources. The maximum specific growth rates ($\mu_{max}$) computed for the first 12 hours of observed growth showed that cells grown on glucose grew approximately twice (1.8 times) as fast as cells grown on glycerol (0.158 vs 0.09 /h) or without carbon added (0.158 vs 0.08 /h), and 5 times faster than those grown

on methanol (0.158 vs. 0.035 /h). The biomass yield of *P. pastoris* on glucose was almost exactly double that obtained on glycerol (0.102 ± 0.026 g/g on glycerol vs. 0.21 ± 0.01 g/g on glucose).

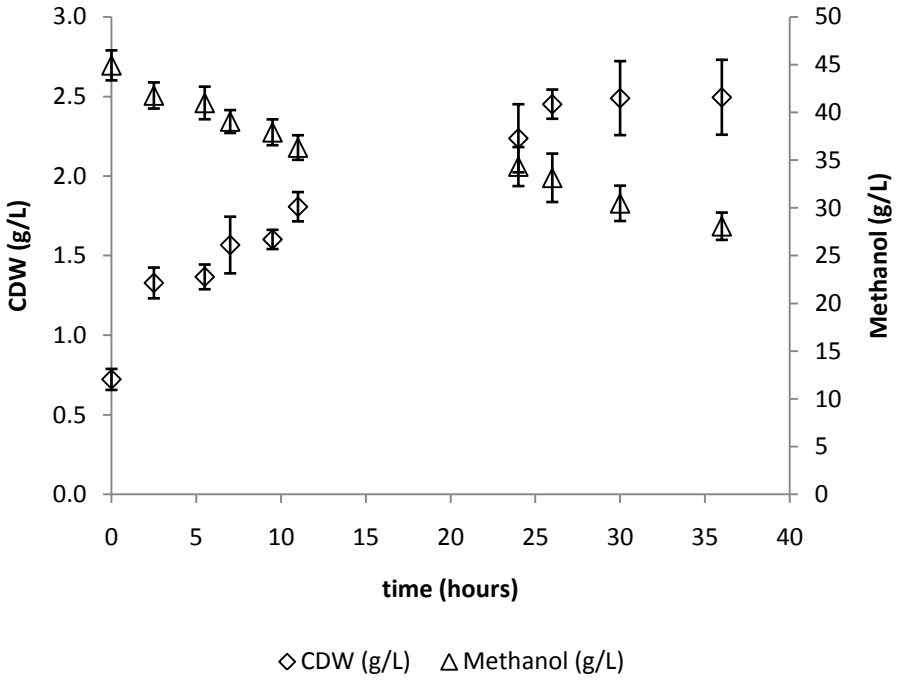


**Figure 4. 3Growth profile of *P. pastoris* in chemically defined medium containing 49 g/L methanol.**

Cells were grown in shake flasks at $30^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights determined via correlation with $OD_{600\ nm}$ (◊); methanol utilization (g/L) (Δ). Results shown are representative of 3 independent experiments and error bars are included.

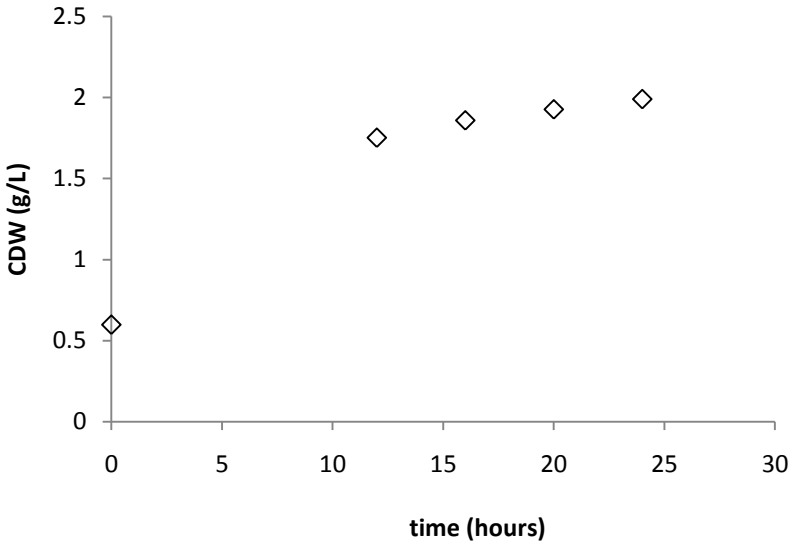


**Figure 4. 4 Growth profile of *P. pastoris* in chemically defined medium without addition of carbon**.
Cells were grown in chemically defined medium having been pre-cultured in complex medium (1 % yeast extract, 2 % peptone) in order to determine the extent of growth due to the yeast extract and peptone carried over in the inoculum contributes.

**Table 4. 1 Summary of kinetic parameters obtained for growth and substrate utilisation parameters obtained following growth of *P. pastoris* in chemically defined media containing glycerol, methanol and glucose**

| | | Growth in chemically defined medium containing carbon | | | |
|---|---|---|---|---|---|
| **Parameter** | **Unit** | **Glycerol** | **Glucose** | **Methanol** | **No added carbon** |
| Maximum specific growth rate[1] ($\mu_{max}$) | /h | 0.09 ± 0.007 | 0.158 ± 0.002 | 0.035 ±0.004 | 0.08 |
| Biomass yield coefficient[2] ($Y_{x/s}$) (apparent) | (g/g) | 0.102 ± 0.026 | 0.21 ± 0.01 | 0.167 ± 0.036 | - |
| Biomass produced[3] (ΔX) | g/L | 4.59 ± 0.08 | 8.40 ± 0.57 | 1.77 ± 0.279 | 1.393 |
| Biomass produced (on added carbon)* (ΔX) | g/L | 3.19 ± 0.08 | 7.01 ± 0.86 | 0.38 ± 0.28 | - |
| Total carbon substrate used | g/L | 50 ± 4 | 39 ± 1 | 44 ± 2 | - |
| Yield on added carbon $Y_{X/S}$ | g/g | 0.06 ± 0.005 | 0.21 ± 0.01 | 0.02 ± 0.01 | - |
| Volumetric substrate uptake rate[4] (dS/dt) | g/Lh | 1.39 ± 0.1 | 0.81 ±0.02 | 0.105 ± 0.02 | - |
| Ethanol yield[5] $Y_{EtOH/glucose}$ | g/g | - | 1.18 ± 0.5 | - | - |
| Rate of ethanol depletion[6] (dEtOH/dt) | g/Lh | - | 1.3 ± 0.3 | - | - |

[1]Calculated by plotting natural logarithm of biomass against time (lnX vs. t) and calculating the gradient over the most linear portion of graph; [2] biomass yield coefficient calculated by dividing total change in biomass (final – initial) by the total change in substrate concentration (initial – final) on added carbon and carbon present in yeast-extract and peptone present in inoculum; [3] total biomass produced (final- initial); [4] substrate uptake rate calculated by dividing change in substrate concentration by change in time; [5]ethanol yield calculated by dividing the amount of ethanol produced per glucose consumed: calculated by dividing the highest ethanol concentration by the amount of glucose consumed at that point. [6] rate of ethanol depletion calculated by dividing the maximum ethanol concentration produced by the time taken for concentration to reach lowest value. Biomass production due to yeast extract and peptone carried over during inoculation was not included for the above calculations. Biomass (actual)* obtained by subtracting the total biomass produced in shake flasks without carbon added from the total biomass produced, calculated according to footnote 3.

Apart from cells cultivated on glucose-containing medium, cells on other carbon sources did not produce ethanol. Ethanol was produced over the first 6 hours of growth and the ethanol yield calculated based upon glucose consumed was 1.18 ±0.5 g/g. However, this exceeds the theoretical maximum yield of 0.51 g/g. This indicates that ethanol production was also supported by carbon-containing materials supplied through the inoculum volume. Subsequently ethanol was used up at a rate of 1.3 ± 0.3 g/L.h. The value for biomass yield on methanol in complex medium (0.167 g/g) is misleading due to the miniscule contribution of methanol to biomass production. Comparing the biomass produced on methanol (1.77 ± 0.279 g/L) to that produced in the control flask in which no carbon was added (1.393 g/L), the contribution of additional carbon (in the form of methanol) to biomass formation (approximately 0.3 g) was shown to be much lower than that of glucose or glycerol (where the total biomass formed on added carbon was 7 and 3.2 g respectively). The calculation for yield coefficient (change in biomass/ change in substrate) thus attributes a large biomass change to a small change in substrate concentration, producing an inordinately high value. To determine the biomass formation due to uptake of the additional carbon, a separate yield calculation was computed as shown in Table 4.1. According to this calculation, biomass yield on glucose was the highest (0.21 ± 0.01 g/g) followed by glycerol (0.06 ± 0.005 g/g). The lowest biomass yield was obtained on methanol (0.02 ± 0.01 g/g), confirming that very little biomass was produced by growth of *P. pastoris* on methanol (between 0.1 and 0.5 g in total). Indeed, the lower growth rate on methanol compared to the control flask (0.035 ± 0.004 /h vs. 0.08 /h on carried over complex nutrients) suggested that residual methanol present at a concentration of 40 g/L may have been inhibitory to the growth of *P. pastoris* in the shake flask experiments conducted.

In general, few studies have attempted to elucidate the fundamental kinetic parameters of *P. pastoris* growth on methanol in shake flask conditions. For $Mut^+$ strains specifically, this is not possible due to the high alcohol oxidase activity (compared to $Mut^S$), which would rapidly degrade methanol, producing hydrogen peroxide and leading to cell lysis and growth inhibition (Sinha *et al.* 2004; Cos *et al.* 2006). However, for this $Mut^S$ strain, while no apparent toxicity was evident (by microscope observation) non-competitive growth inhibition may have occurred due to the slow rate of methanol breakdown owing to the strain possessing only 10 % the AOX activity of the wild type strain. The growth rate obtained (0.035±0.004 /h) was approximately 44 % that obtained in the control flask (0.08 /h) where no carbon source was added. On comparing growth on glucose to that on glycerol, the higher maximum specific growth rate, biomass yield and final biomass produced on glucose suggest it as the carbon source of choice for subsequent large-scale experiments. However, the production of ethanol during growth on glucose has critical importance in evaluating its

potential use in subsequent bioreactor studies due to the repressive effect of ethanol on the AOX promoter (Inan, Meagher 2001a). The mechanism by which ethanol represses the AOX promoter has not been thoroughly studied; however Inan *et al.*(2001) suggested that it does this by catabolite inactivation, the process by which blocking a carbon transporter in the cell membrane prevents the carbon compound entering the cell, resulting in the impairment of the cell's physiology (Riballo *et al.* 1995). Additionally, they found that glucose (and glycerol) was responsible for catabolite repression. Repression prevents the synthesis of alcohol oxidase enzymes and inactivation results in degradation of enzymes produced. Thus, despite the favourable growth characteristics of glucose, the potential for enzyme inactivation in a fed-batch process indicated glycerol as the carbon source of choice for subsequent scaled-up experiments.

### 4.2.3 Growth in complex media containing methanol, glucose and glycerol

Growth was investigated in a complex medium containing 1% yeast extract and 2% peptone (collectively known as YP) supplemented with glucose, glycerol or methanol. As a control, to determine the additional contribution to biomass production of carbon compounds added to YP, cells were grown in YP medium alone.

Figure 4.5 shows *P. pastoris* growth on YP alone. There was an initial lag phase during the first two hours after inoculation. Between the 2$^{nd}$ and 10$^{th}$hour, growth increased linearly at a $\mu_{max}$ of 0.12 ± 0.04 /h. Beyond the 10$^{th}$ hour, the μ decreased (= 0.01 /h). Between 12 and 24 hours of growth, the biomass did not increase suggesting that cells reached stationary phase, probably due to nutrient limitation.

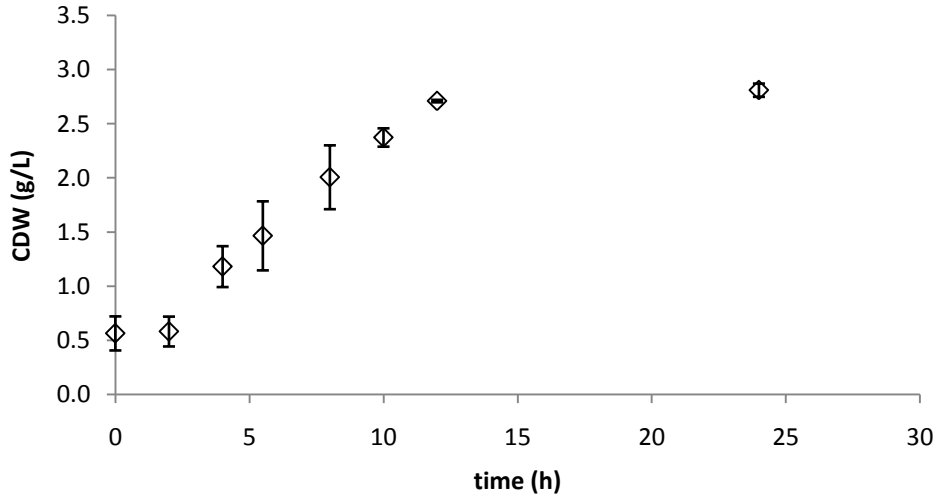


**Figure 4. 5Growth profile of *P. pastoris*in complex YP medium (with no carbon source added).**
Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) determined via correlation with $OD_{600\ nm}$ (◊); results shown are representative of three independent experiments.

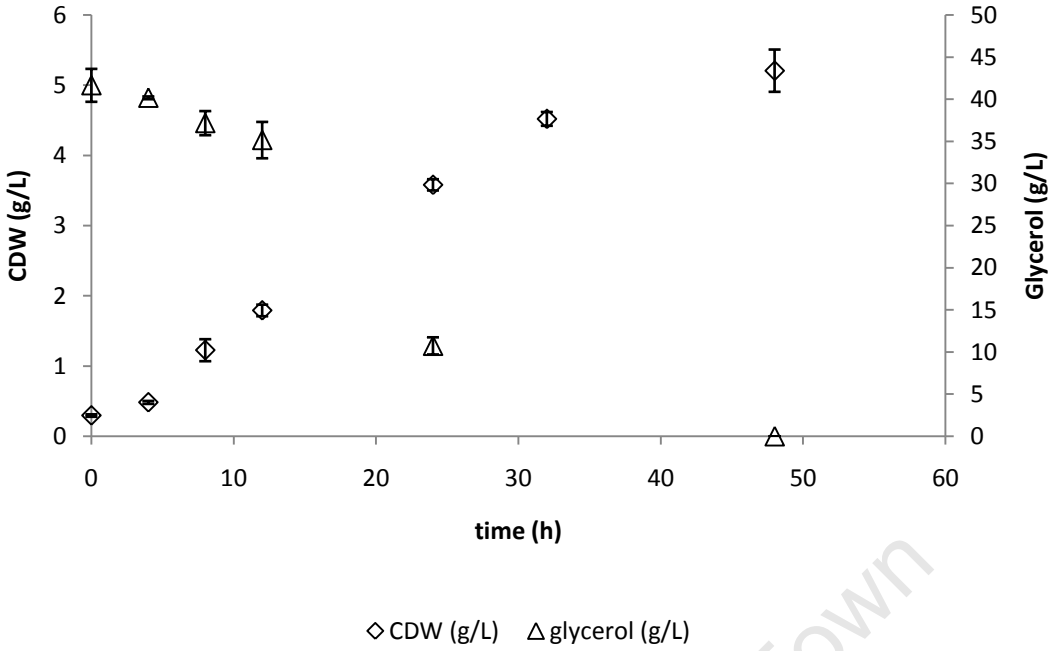


**Figure 4. 6 Growth profile of *P. pastoris*in complex YP medium with 41 ± 2 g/L glycerol added.**
Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) weredetermined via correlation with $OD_{600\ nm}$ (◊); glycerolutilisation (g/L) (Δ) shown above isfrom three independent experiments (error bars included).

Figure 4.6 shows the growth of *P. pastoris* on YP-glycerol with a starting glycerol concentration of 41 ± 2 g/L. There was no identifiable lag phase in these flasks, as exponential growth was achieved over the first 8 hours of growth at a maximum specific growth rate of 0.18 ± 0.02 /h. The specific growth rate decreased after 8 hours, from a μ value of 0.18 ± 0.02/h to 0.1 ± 0.02/h. The rate of glycerol utilisation over the initial phase of growth (0-12 hours) was 0.5 ± 0.3 g/L.h and increased to 2 ± 0.2 g/L.h during overnight incubation over the next 12 hours. The subsequent biomass change during this period of rapid substrate utilisation was smaller (1.8 ± 0.007 g/L) compared to the biomass formed during the first 12 hours. This suggests the formation of an intermediate extracellular compound resulting from glycerol metabolism. However, the substrate analyses via HPLC did not reveal the formation of acetate or ethanol, typical by-products of glycerol cultivation and repressors of the AOX promoter. Alternatively, these data suggested that growth may have been supported by yeast extract and peptone during the first 12 hours of growth and that subsequent nutrient limitation occurred. The specific growth rate (dX/dt) was roughly linear between 12 and 40 hours which may be indicative of growth being limited by oxygen supplied at a constant rate. As growth progressed, the dissolved oxygen concentration approached zero, resulting in linear growth. However, the higher linear rate of growth maintained in the glucose flask (Figure 4.7) suggested that this might not be the case.

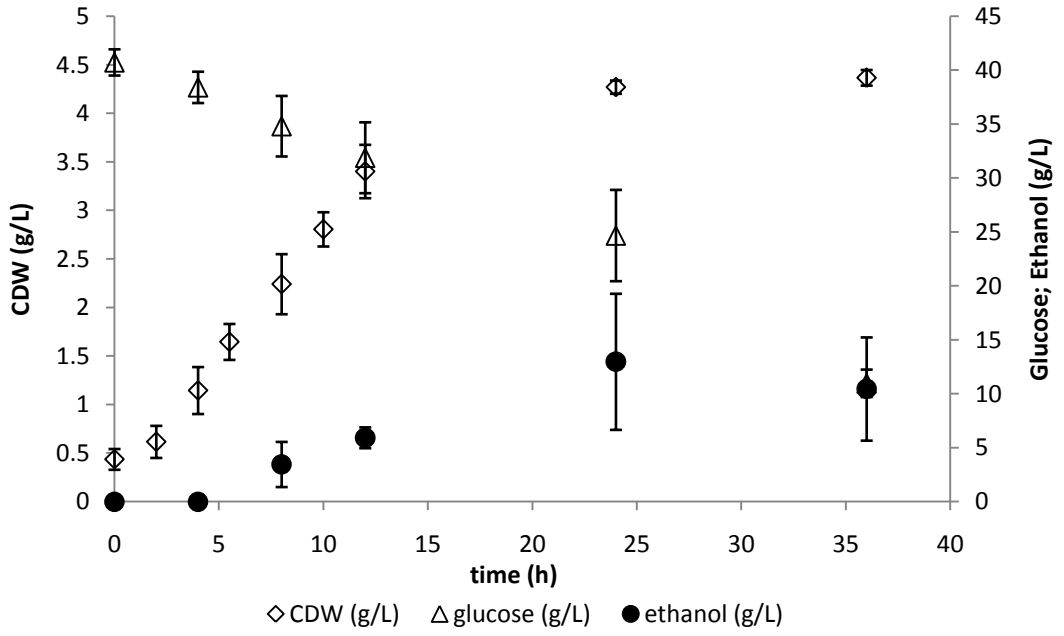


**Figure 4. 7Growth profile of *P. pastoris* complex YP medium with 40 ± 1g/L Glucose added**.

Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) were determined via correlation with $OD_{600\ nm}$ (◊); Glucose utilisation (g/L) (Δ) and Ethanol production (•) shown above are from three independent experiments (error bars included)

Figure 4.7 shows growth on YP-glucose, with glucose present at a starting concentration of 40 ± 1 g/L. Cells grew exponentially upon inoculation, with a maximum specific growth rate, of 0.21 ± 0.02 /h maintained over the first 8 hours of growth. The biomass produced during this phase was 3.4 ± 0.27 g/L. *P. pastoris* utilised glucose at a rapid rate (at an average rate of 0.7 g/Lh) upon inoculation. During growth over 24 hours, some glucose in the medium was metabolized to produce ethanol in addition to biomass. The ethanol concentration increased to 10 ± 4 g/L at t = 24 h, remaining stable at this concentration until the end of the experiment at 48 hours. The cells entered stationary phase before 24 hours of growth despite the high availability of residual carbon in the medium. Thus growth may have occurred at a linear rate between 5 and 12 hours due to lack of a non-carbon limiting substrate.

Figure 4.8 shows the biomass production and methanol utilisation observed when *P. pastoris* was cultivated in YP medium containing methanol at a starting concentration of 43.1 ± 1.9 g/L. The biomass produced during the first 12 hours of growth (1.45 ± 0.15 g/L) with a $\mu_{max}$ value of 0.12 ± 0.04 /h was lower than that produced during the first 12 hours' growth on glucose (1.87 ± 0.1 g/L), but not statistically different to that produced on glycerol (1.5 ± 0.07 g/L).

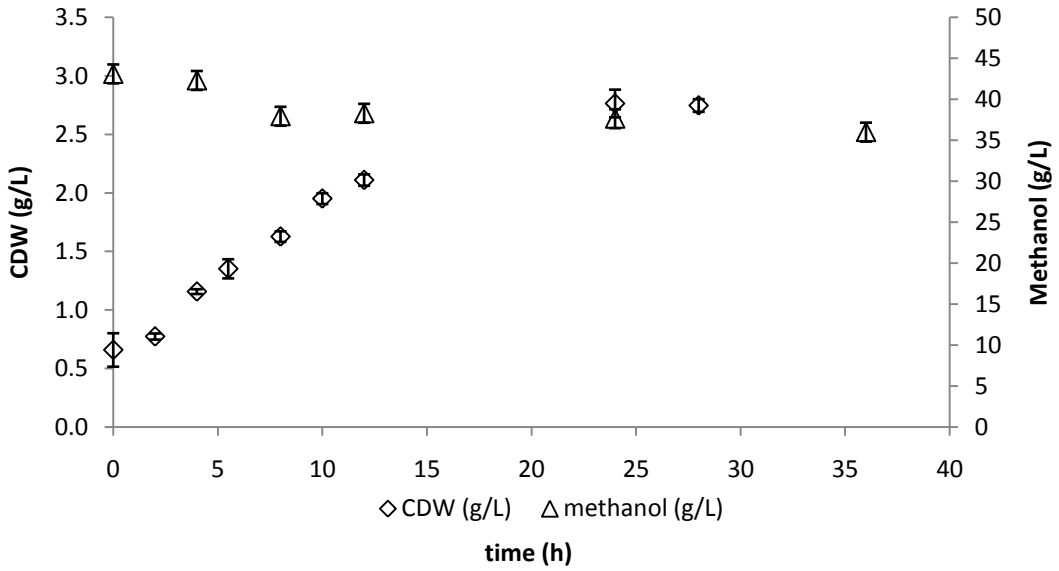


**Figure 4. 8Growth profile of *P. pastoris* complex YP medium with 43 ± 2 g/L methanol added.**

Cells were grown in shake flasks at 30$^0$C and 150 rpm agitation on an orbital shaker. Cell dry weights (CDW) determined via correlation with $OD_{600\ nm}$ (◊); Methanol utilization(g/L) (Δ) from three independent experiments is shown (error bars included).

Cells appear to enter stationary phase after 24 hours of cultivation. The rate of methanol depletion was highly variable among the flasks (0.2 ± 0.2 g/Lh). Accounting for the rate of methanol evaporation, only one flask appeared to show any methanol utilisation (at a rate of 0.01 g/Lh). It can therefore be concluded that little methanol utilisation occurred.

Table 4.2 compares the growth obtained on YP-medium with and without additional carbon sources. Supplementing carbon present in the complex YP medium with glucose led to a dramatic increase in specific growth rate and biomass production. The higher biomass concentrations achieved upon addition of glycerol and glucose indicates that while rapid growth occurs on YP-alone, this growth is limited due to lack of carbon availability following depletion of the complex hydrolysates present in YP.

The biomass produced in the methanol-containing media (2.0 ± 0.09 g/L) was similar to biomass concentration produced on YP-alone (2.24 ± 0.21 g/L). The decrease in methanol was largely attributable to evaporation (average rate of depletion: 0.262 g/Lh, rate of evaporation in shake flask: 0.294 g/Lh). Thus the biomass production was supported by yeast-extract and peptone only. The substrate uptake rates obtained upon glycerol and glucose were similar (0.87 ± 0.03 vs. 0.83 ± 0.06 g/Lh) in chemically defined medium. However, ethanol was produced upon growth on glucose. The biomass produced on the additional carbon was analysed as for the chemically defined medium and is presented in Table 4.2. .

**Table 4. 2 Growth kinetic parameters obtained upon cultivation of *P. pastoris* in a complex medium**

| | | Growth in complex medium containing carbon | | | |
|---|---|---|---|---|---|
| **Parameter** | **Unit** | **Glycerol** | **Glucose** | **Methanol** | **Media alone (control)** |
| **Maximum specific growth rate($\mu_{max}$)[1]** | /h | 0.18 ± 0.02 | 0.173 ± 0.03 | 0.12 ± 0.04 | 0.134 ± 0.022 |
| **Biomass yield coefficient (Yx/s)[2]** | (g/g) | 0.12 ± 0.006 | 0.13± 0.002 | [c]0.21 | - |
| **Biomass produced (ΔX)[3]** | g/L | 3.93 ± 0.19 | 4.91 ± 0.31 | 2.09 ± 0.09 | 2.24 ± 0.21 |
| **Biomass produced on added carbon*** | (g) | 1.69 ± 0.19 | 2.67 ± 0.31 | - | - |
| **Biomass yield on added carbon **** | (g/g) | 0.06 ± 0.003 | 0.09 ± 0.001 | - | |
| **Volumetric substrate uptake rate (dS/dt)[4]** | g/Lh | 0.87 ± 0.04 | 0.83±0.06 | [c]0.262 | - |
| **Ethanol yield $Y_{EtOH/glucose}$[5]** | g/g | - | 0.576 ± 0.17 | | - |
| **Rate of ethanol depletion (dEtOH/dt)[6]** | g/Lh | - | 1.0 ± 0.30 | 0.09 ± 0.05 | - |

1 calculated by plotting natural logarithm of biomass against time (lnX vs. t) and calculating the gradient over the most linear portion of graph;
2 biomass yield coefficient calculated by dividing total change in biomass (final – initial) by the total change in substrate concentration (initial – final);
3 total biomass produced (final- initial);
4 substrate uptake rate calculated by dividing change in substrate concentration by change in time;
5ethanol yield calculated by dividing the amount of ethanol produced per glucose consumed: calculated by dividing the highest ethanol concentration by the amount of glucose consumed at that point.
6rate of ethanol depletion calculated by dividing the maximum ethanol concentration produced by the time taken for concentration to reach lowest value; Biomass production due to yeast extract and peptone carried over during inoculation was not included for the above calculations.
c results obtained from a single experiment.
*subtracted 2.24 g from each of the values obtained on glycerol, glucose and methanol.
**divided the biomass produced by total substrate consumed during growth.

From these values the biomass yield coefficient upon carbon was computed. No methanol values were obtained because upon subtracting the biomass production attributable to complex medium, there is no biomass production on methanol, suggesting poor uptake of methanol. The low methanol uptake rate could be attributed to a several reasons. Firstly, this particular $Mut^S$ strain had a lower alcohol oxidase activity than the wild type strain ($Mut^+$) therefore absorbed and utilised methanol more slowly. Secondly, methanol utilisation is a highly aerobic process with a high oxygen requirement. Shake flasks, characterized by a lower oxygen transfer rate than bioreactors, therefore do not supply sufficient oxygen required for methanol breakdown (Jungo *et al.* 2007). A compound present in yeast extract and peptone may have repressed the AOX gene, preventing methanol utilisation in the presence of complex medium. A comparison of the parameters obtained in chemically defined and complex medium is presented in Table 4.3.

### .2.4 Discussion

Table 4.3 compares data across the shake flask experiments conducted, combining data from Tables 4.1 and 4.2. Generally higher $\mu_{max}$ values were obtained in complex media than in chemically defined media. Yeast extract and peptone contain a variety of vitamins, carbon compounds such as acetyl coA, biotin, nicotinic acid (a precursor to NAD and NADP-key cofactors in biomass production), various amino acids and hydrolysates of animal protein that aid the growth of *P.pastoris* (ATCC Catalogue of bacteria and bacteriophage, 1989). These compounds are readily absorbed and utilised in existing form with little need for enzymatic processing. In chemically defined media, the nutrients present (ammonium, glycerol, various trace elements) must be absorbed following the synthesis of enzymes for their uptake and hydrolysis. The metabolic pathway activation required to convert substrate to biomass takes longer than in complex medium.

In order to further elucidate the differences in growth, the carbon uptake rates in molar quantities are shown in Table 4.4.

**Table 4. 3Comparison of the molar carbon uptake rates across glucose and glycerol containing media**

| Substrate uptake rate | YP-glycerol | YP-glucose | Chemically defined (glycerol) | Chemically defined (glucose) |
|---|---|---|---|---|
| **g/Lh** | 0.87 ± 0.04 | 0.83 ± 0.06 | 1.39 ± 0.1 | 0.81 ± 0.02 |
| **Mol Carbon/h** | 0.03 ± 0.04 | 0.027 ± 0.06 | 0.027 | 0.03 |

**Table 4. 4Comparison of growth kinetic parameters in chemically defined and complex medium. The data from chemically defined and complex media have been combined below (footnotes identical to those provided in tables 4.1 and 4.2)**

| | | Complex medium | | | | Chemically defined medium | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | **Unit** | **YP-alone** | **YP-glycerol** | **YP glucose** | **YP methanol** | **Glycerol** | **Glucose** | **Methanol** | **Media alone (control)** |
| Maximum specific growth rate ($\mu_{max}$ ) | /h | 0.134 ± 0.02 | 0.18 ± 0.02 | 0.173 ± 0.03 | 0.12 ± 0.04 | 0.09 ± 0.007 | 0.158 ± 0.002 | 0.035 ± 0.004 | 0.08 |
| Biomass yield coefficient ($Y_{X/S}$) | g/g | | 0.12 ± 0.006 | 0.13 ± 0.002 | [c]0.21 | 0.102 ± 0.026 | 0.21 ± 0.01 | 0.1 ± 0.02 | - |
| Biomass produced (total) | g | 2.24 ±0.21 | 3.93± 0.19 | 4.91 ± 0.31 | 2.09± 0.09 | 4.59 ± 0.08 | 8.40 ± 0.57 | 1.77 ± 0.279 | 1.393 |
| Biomass production on added carbon | g | - | 1.69 ± 0.19 | 2.67 ± 0.31 | - | 3.19 ± 0.08 | 7.01 ± 0.86 | 0.38 ± 0.28 | |
| Biomass yield on added carbon | g/g | - | 0.06 ± 0.003 | 0.09 ± 0.001 | - | 0.06 ± 0.005 | 0.21 ± 0.01 | 0.02 ± 0.01 | |
| Volumetric rate of substrate uptake (dS/dt) | g/Lh | - | 0.87 ± 0.04 | 0.83 ± 0.06 | [c]0.01 | 1.39 ± 0.1 | 0.81 ± 0.02 | 0.105 ± 0.02 | - |
| Volumetric rate of ethanol production (dS/dt) | g/Lh | - | - | 0.5 ± 0.28 | - | - | 1.3 ± 0.3 | - | - |

As shown in the Table 4.4, the relative uptake rates in terms of moles carbon absorbed per hour were very similar in both types of media. Despite this, the biomass production was significantly higher in cells cultivated in glucose containing medium than on glycerol. Indeed, in chemically defined medium the biomass production on glucose was approximately 1.8 times that produced on glycerol. Given the similar carbon uptake rates this result would suggest that glycerol was diverted to the production of an intermediate compound. However, substrate analyses for acetate and ethanol, the key intermediate compounds in the glycerol metabolic pathway, revealed that neither was being produced. Thus it was speculated that glycerol might have been converted to an extracellular storage product, thus accounting for its rapid uptake rate but slow biomass production rate. Given the respiratory nature of *P. pastoris* growth on glycerol, it is additionally possible that the unaccounted carbon might have been directed towards carbon dioxide production. While there have been no shake flask studies conducted to investigate the metabolic flux of glycerol and glucose through *P. pastoris*, chemostat experiments have been performed. Sola *et al.*(2004) used two different dilution rates with radio-labelled glucose and glycerol, in order to assess their metabolic pathways. They found that for the same dilution rate (0.16 /h), a higher quantity of $CO_2$ and a lower quantity of biomass was produced on cells grown on glucose than on cells grown on glycerol, directly opposite to this thesis' observations. No ethanol or acetate production was noted in their experiments on either carbon source. In order to verify whether a similar finding would be obtained in the batch shake flask experiments conducted therefore, a radio-labelling approach (of glucose and glycerol) could be adopted for these experiments.

Cells grown on glucose in both defined and complex media produced ethanol. The ethanol yield on glucose and the ethanol production rate was higher in the chemically defined medium compared to the complex medium. Ethanol production is part of the glucose and glycerol breakdown pathway. The rapid absorption of glucose and its conversion to biomass could be accounted for by the fact that *P. pastoris* is endowed with 14 hexose transporters on its membrane which therefore allow for rapid glucose uptake (Mattanovich *et al.* 2009).

Growth on YP-methanol was faster than in chemically defined medium containing methanol (0.12 ± 0.04 /h vs. 0.035 ± 0.004 /h, respectively). This could be attributed to the additional nutrients supplied to support growth. The methanol uptake rate was higher in chemically defined medium compared to complex medium where methanol uptake was negligible. The final biomass produced was higher in complex medium than in chemically defined medium (2.09 ± 0.09 g/L vs. 1.77 ± 0.279 g/L). The contribution to biomass production due to yeast extract and peptone could be assessed by comparing the growth in chemically defined medium containing methanol to the growth in

chemically defined medium with no carbon source added. The additional biomass attributable to methanol is approximately 0.38 ± 0.28 g/L. For complex media, the biomass attributable to methanol was negligible. While the oxygen demand in the presence of methanol has been cited as a constraint, this cannot fully explain the slow growth observed under conditions of this study. A comparison between the growth rates of *P. pastoris* on YP-methanol and YP-alone (0.08 ± 0.008 /h vs. 0.134 ± 0.002 /h) and between chemically defined medium without methanol (0.08 /h) and with methanol added (0.035 /h) shows that addition of methanol to the medium appeared to inhibit the growth. The growth inhibitory effect of methanol on $Mut^+$ strains is widely reported in the literature (Cos *et al.* 2006a). Zhou and co-workers (2002) demonstrated that this inhibition follows a non-competitive growth inhibition model (Zhou, Zhang 2002). According to Monod kinetics for non-competitive inhibition of growth, above a critical substrate concentration $S_{crit}$, the maximum specific growth rate $\mu_{max}$ decreases with increasing substrate concentration. Thus it is probable that the concentration used to investigate growth on methanol was above $S_{crit}$. A more detailed investigation of the effect of methanol concentration on growth (and protease expression) is presented later in this study.

Thus, from the shake flask data, it was concluded that despite the higher biomass production in shake flasks containing glucose, the production of ethanol during growth made it unsuitable for scaled-up processes. While methods of overcoming the limitations of using glucose as a carbon source are discussed in Section 5.2, a cost analysis of glucose and glycerol revealed that glycerol is a cheaper carbon compound than glucose. According to most recent price lists available from Merck, glucose costs ZAR 82 per kilogram compared to glycerol which costs ZAR 42 per kilogram (Merck, 2011). This significant cost difference further established glycerol as the carbon source of choice for subsequent bioreactor experiments.



## 4.3Batch growth kinetics of *P. pastoris* in chemically defined and complex media containing glycerol determined in bioreactor experiments

### 4.3.1 Growth kinetics in complex media

*P. pastoris* was cultivated in a New Brunswick BioFlo 110 bioreactor in complex media containing 40 g/L glycerol (reactor configuration and inoculum preparation described in Sections 3.5 and 3.6). Figure 4.9 shows the growth and substrate utilisation trajectories obtained. Initially, from approximately the first hour to the fifth hour, biomass increased from 0.28 to 0.44 g/L at a specific growth rate (μ) of 0.111 /h. During this period the measured glycerol concentration showed a slight decrease from approximately 42 g/L to 41 g/L. From the fifth hour to the $12^{th}$ hour, exponential growth was observed, with a $\mu_{max}$ of 0.288 /h being achieved and the glycerol concentration

decreased from 40 to 26 g/L over this period. Subsequently, cells grew at a slower growth rate of 0.076 /h from the 12$^{th}$ to the 36$^{th}$ hour. During this period cells utilised the remaining glycerol, with the concentration decreasing from 26 g/L to approximately 1 g/L.

The glycerol utilisation rate over the first seven hours of growth was approximately 1 g/Lh. The utilisation rate increased to 2.2 g/Lh during the exponential phase of growth (from t = 7 h to t = 26 h). Similarly the biomass yield obtained upon glycerol during the first seven hours of growth was 0.24 g/g, increasing to 0.4 g/g from the 7$^{th}$ to the 26$^{th}$h. The inoculum prepared for this experiment was pre-cultured on YP-glycerol for 24 hours therefore it is unlikely that the significant differences in glycerol uptake were due to adaptation to glycerol as the inoculum was well adapted. It was postulated that the initial period of growth occurred upon yeast-extract and peptone in the medium together with glycerol.

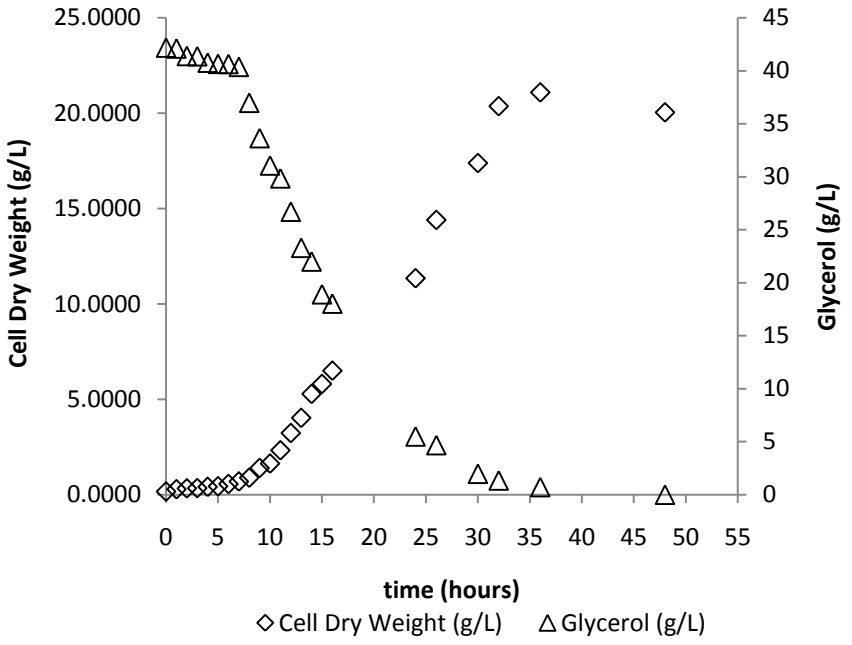


**Figure 4. 9 Bioreactor experiment to investigate the growth profile of *P. pastoris* in complex media containing 44 g/L glycerol.** Complex media (yeast-extract 1%; peptone 2 %) with 40 g/L glycerol added in the presence of 1 M Phosphate buffer (10%v/v). The reactor was run at 30$^0$C at 600-800 rpm. Glycerol concentration(Δ) and biomass concentration(◊)from a single bioreactor experiment are shown.

To investigate the biomass production attributable to YP, a separate growth experiment on YP was performed. Cells (precultured on YP) were added to buffered YP medium in a bioreactor and growth monitored. The results are shown in Figure 4.10. Additionally, the growth obtained on YP-glycerol is

super-imposed on the graph. Cells cultivated in YP alone did not display any significant lag phase. Upon inoculation, *P. pastoris* achieved a $\mu_{max}$ of 0.187 /h during exponential phase. Cells abruptly entered stationary phase after 12 hours of growth, the biomass concentration increasing only slightly from 1.36 g/L at t = 12 h to 1.42 g/L at t = 24 h. Thus the total biomass produced on YP-alone was 1.25 g/L. Comparing the growth profiles on YP-alone and YP-glycerol over the first seven hours of growth, the biomass produced on YP-glycerol was 0.52 g/L whereas the biomass production on YP-alone was 0.58 g/L. Taken together, the data suggest that in the presence of YP, glycerol utilisation is slow. Upon depletion of carbon in yeast-extract and peptone, utilisation of glycerol increased.

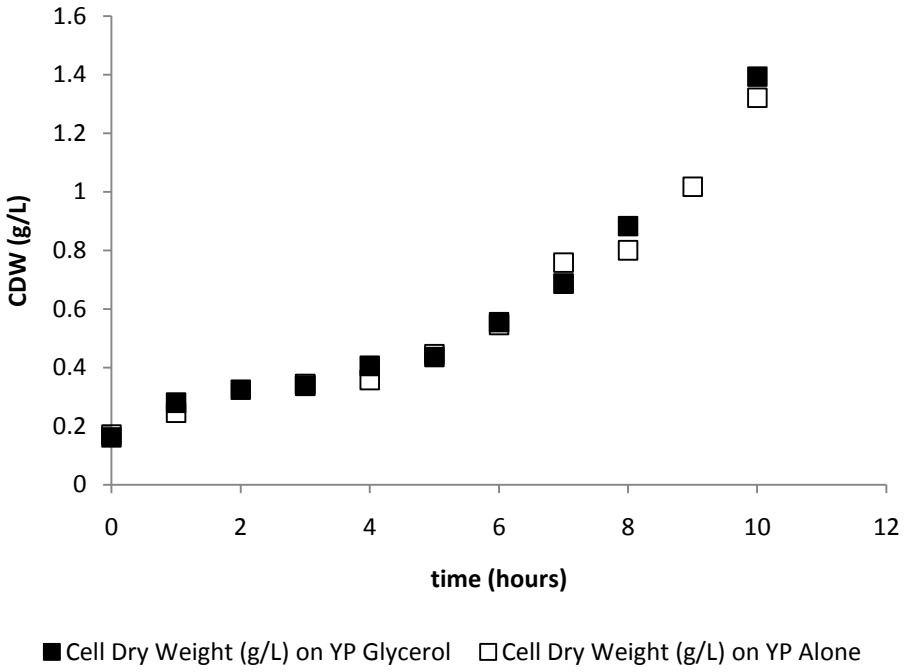


**Figure 4. 10 Bioreactor experiment to determine growth profile of *P. pastoris* on yeast peptone alone.**
Cells were grown on yeast-extract (1%); peptone (2%) for 24 hours. Data was obtained from a single experiment. The growth obtained during the first phase of growth on YP-glycerol has been super-imposed, indicating the biomass production likely to be attributable to yeast-peptone during early growth.

For the subsequent fed-batch experiments the slow utilisation of glycerol in the presence of carbon contained in yeast extract and peptone would be problematic, given the need to maintain exponential growth by supplying the nutrient in a growth-limiting manner during the fed-batch phase. Supplying YP together with glycerol may depress glycerol utilisation as cells deplete carbon in YP before using glycerol. This would result in the accumulation of glycerol in the reactor, possibly leading to substrate inhibition via metabolic overflow. Thus growth and substrate utilisation were investigated in chemically defined medium with glycerol as a carbon source.

### 4.3.2 Growth kinetics on chemically defined media in bioreactor experiments

Cells were pre-cultured on YP containing glycerol as described in Section 3.3.2. Figure 4.11 shows the growth and substrate utilisation profile of *P. pastoris* in chemically defined medium (composition given in Section 3.3.1) over triplicate experiments. During the first eight hours, biomass increased slightly, from 0.57 ± 0.1 g/L at inoculation to 1.6 ± 0.3 g/L at the 8$^{th}$ hour at a growth rate of 0.12 ± 0.03 /h. The biomass yield upon glycerol during the first 8 hours of growth was 0.11 ± 0.01 g/g. From t = 12 h to t = 20 h, cells grew faster, achieving a $\mu_{max}$ of 0.21 ± 0.01 /h. During exponential growth, biomass increased from 2.19± 0.3 g/L at the 12$^{th}$ hour to 25.2 ± 2.1 g/L at the end of 24 hours at an average maximum specific growth rate of 0.21 ± 0.018 /h. During this period, glycerol decreased from 39 ± 3 g/L to 5 ± 1 g/L and the dissolved oxygen levels fell from a saturated concentration value of 6.7 ± 0.3 mg/L at t = 7 h to 0.1 ± 0.07 mg/L at t = 22 h. The dissolved oxygen concentration in mg/L was calculated following the measurement as a percentage saturation of oxygen as a partial pressure of 0.21 atm in distilled water at 30$^0$C. The saturation concentration under these conditions was taken from Bailey and Ollis(1977).The average rate of substrate uptake during exponential phase was 2.8 g/Lh which was comparable to the exponential rate of substrate uptake in YP-glycerol (2.2 g/Lh) shown in Figure 4.9.



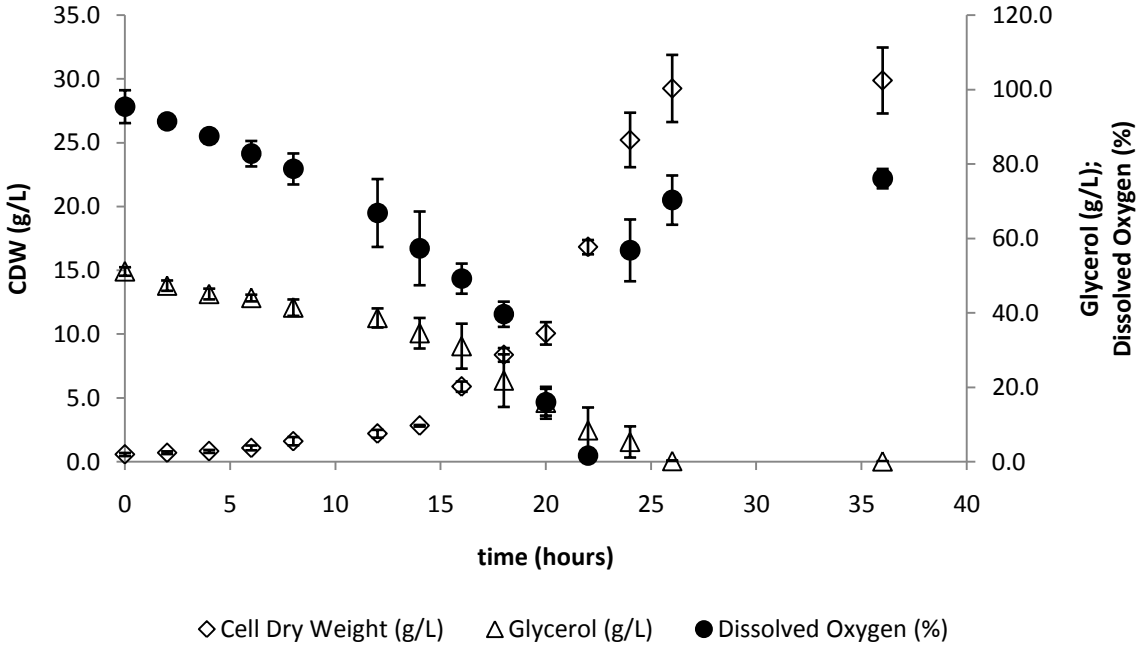


**Figure 4. 11Bioreactor experiment to determine the growth profile of *P. pastoris* in chemically defined media containing 50 g/L glycerol.**
Data shows biomass concentration(◊), substrate concentration (Δ) and dissolved oxygen concentration (•). Data was obtained from threeindependent experiments.

**Table 4. 5 Analysis of kinetic growth parameters for cells cultivated in bioreactors in chemically defined and complex media containing glycerol (* single experiment)**

| | | Growth in shake flasks | | Growth in bioreactors | |
|---|---|---|---|---|---|
| **Parameter** | **Unit** | **Complex** | **Chemically defined** | **Complex*** | **Chemically defined** |
| **Maximum specific growth rate $\mu_{max}$[1]** | /h | 0.088 ± 0.015 | 0.09 ± 0.007 | 0.235 | 0.21 ± 0.02 |
| **Biomass yield coefficient, $Y_{X/S}$[2]** | g/g | 0.12 ± 0.006 | 0.102 ± 0.026 | 0.5 | 0.54 ± 0.01 |
| **Biomass produced, $\Delta X$[3]** | g | 3.9 ± 0.2 | 4.5 ± 0.08 | 20.9 | 29.1 ± 0.45 |
| **Volumetric rate of substrate uptake dS/dt[4]** | g/Lh | 0.87 ± 0.04 | 1.39 ± 0.1 | 1 | 1 ± 0.04 |

1.Gradient of graph obtained when natural logarithm of biomass .(Ln(biomass)) is plotted against time2. Change in biomass/ change in substrate = Final biomass- initial biomass/(initial substrate conc-final substrate conc)3.Final biomass-initial biomass 4.Change in substrate concentration/change in time (initial – final substrate conc)/(final – initial biomass conc*Experiment done once

### 4.3.3 Discussion of data obtained on chemically defined media

Table 4.5 compares the growth kinetic parameters obtained in the shake flask and bioreactor studies. Biomass production in bioreactors was vastly superior to biomass production in shake flasks. The final biomass produced in bioreactors averaged 5.9-fold higher than that in shake flasks. The specific growth rate in both media was an average of 4.7-fold higher in the bioreactor compared to shake flasks. Little change in substrate utilisation was observed. In general, the rate of substrate conversion to biomass was much higher in bioreactors than in the shake flask experiments conducted. The higher biomass produced in the bioreactors was likely due to the higher rate of mixing, ensuring a higher availability of nutrients and dissolved oxygen as well as the pH and temperature control.

Important differences were noted between bioreactor experiments in complex vs. chemically defined media. As shown in Table 4.5, the final quantity of biomass produced in chemically defined medium was higher (29.1± 0.45 g/L vs. 20.9 g/L in complex medium containing glycerol). Thus, despite the slight differences in maximum specific growth rates (0.21 ± 0.02 /h in chemically defined vs. 0.235 /h in complex media), the higher biomass yield obtained in chemically defined medium was favourable. It was selected as the growth medium for the fed-batch study.

## 4.4 Kinetic parameters compared to literature values

The growth rates and yield coefficients obtained in the bioreactor in chemically defined medium containing glycerol were compared to literature values obtained from other $Mut^S$ strains of *P. pastoris* using an identically constituted growth medium (Anjou & Daugulis 2001; Hang *et al.* 2009; Gurramkonda *et al.* 2010) in Table 4.6 and to an insulin-producing $Mut^+$ strain.

**Table 4. 6Comparison between kinetic parameters obtained and literature values**

| Parameter | This study | D'Anjou *et al.* (2001) | Hang *et al.* (2010) | *Gurramkonda *et al.* (2010) |
|---|---|---|---|---|
| $\mu_{max}$ (/h) | 0.22 ± 0.08 | 0.26 | 0.18 ± 0.02 | 0.17 |
| $Y_{x/s}$ (g/g) | 0.55 ± 0.01 | 0.4 | 0.45 ± 0.04 | 0.61 |
| Strain | $Mut^s$ | $Mut^s$ | $Mut^s$ | $Mut^+$ |

*insulin producing strain

The growth rates and biomass yields obtained were comparable to those in the literature obtained on the same *P. pastoris* strain. The growth rates obtained by Gurramkonda *et al.*(2010)and Hang *et al.*(2010) were slightly lower (0.17 compared to 0.22 /h), despite the biomass yields being higher than those obtained in this study (0.61 vs. 0.55 g/g). Thus, kinetic parameters obtained in this study were similar to those obtained with a similar strain but differed to those obtained on the $Mut^+$ strain used by Gurramkonda *et al.* (2010).

From Table 4.6, it can be seen that the growth rates obtained in this study were comparable to those reported in the literature. However, the biomass yield obtained by Gurramkonda *et al.*(2010) in the $Mut^+$ strain was higher than that obtained in this study, whereas biomass yields obtained with $Mut^S$strains were lower.

## 4.5 Optimisation of the methanol induction phase

### 4.5.1 Introduction

Having established the optimal batch and fed-batch kinetic parameters, the methanol induction phase parameters were established. The complications involving the use of methanol to induce gene expression in *P. pastoris* have been outlined in Section 2.9.2 as well as the risks of using the flammable substance. Thus it was necessary to determine a methanol concentration that did not lead to a high protease activity and that did not inhibit cell growth.

### 4.5.2 The effect of methanol concentrations on protease production in *P. pastoris*

The first experiment investigated the effect of methanol concentration on protease activity. Cells grown in YP- glycerol (40 g/L) were cultivated for 48 hours (at which point glycerol was depleted) followed by the addition of methanol at various concentrations for another 24 hours before quantifying the protease activity. The assay was calibrated according by correlating the spectrophotometric change produced by known concentrations of proteinase K (see Appendix F). From the linear relationship obtained, the absorbance measurement allowed the determination of the protease activity.

Figure 4.12 shows that increasing protease activity was obtained at increasing the residual methanol concentrations. The highest protease activity recorded was 0.076 units/mg in cells grown at 40 g/L residual methanol. Some background protease activity (0.03 units/mg) was recorded in the control flask which contained complex medium without any glycerol added (with no methanol added). The change in protease activity as the residual methanol concentration increased from 1 to 4 g/L was from 0.03 to 0.05 units/mg. As the residual methanol concentration increased to 10 g/L protease activity increased only slightly from 0.056 to 0.057 units/mg and at 20 and 40 g/L was 0.068 and 0.076 units/mg respectively.



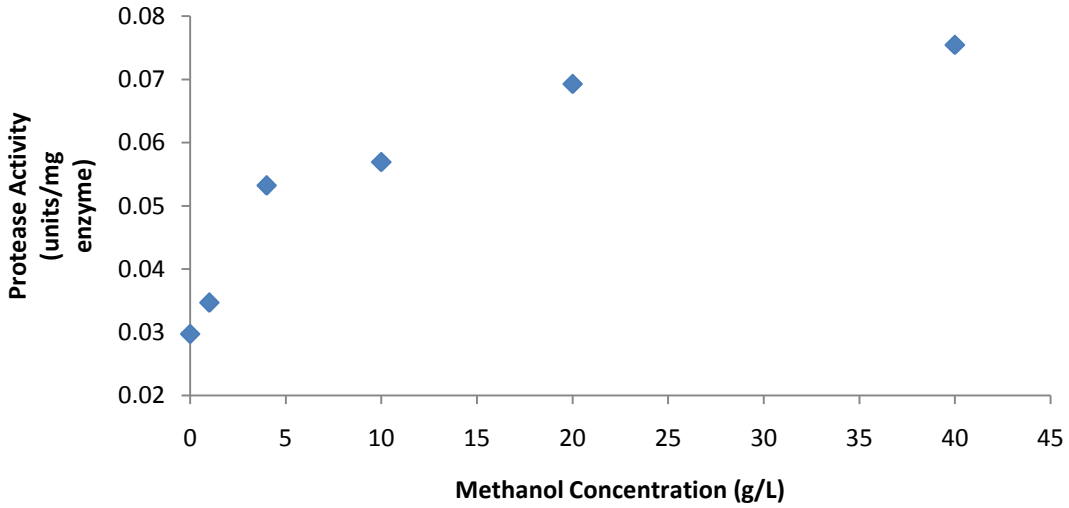


**Figure 4. 12The effect of methanol on protease production.**

Cells were grown in shake flask cultures on YP-glycerol for 48 hours; subsequently cells were incubated in methanol at various concentrations for 24 hours. Supernatant samples were harvested from each of the flasks and protease activity determined. Results are representative of a single experiment

The exact mechanism for methanol concentration dependent protease release is not entirely known. In a bioreactor study on protease activity Sinha *et al.*(2005) observed a significantly higher protease activity of 2888 units/mg protein when *P. pastoris*Mut$^{+}$ strain was grown in a batch bioreactor on methanol as the sole carbon source. The lower activity observed in the Mut$^{S}$ strain used in this study was likely due to the lack of the AOX 1 enzyme reportedly responsible for up to 80% of the total cellular alcohol oxidase activity (Sinha *et al.* 2005). As discussed in Section 2.4.1, hydrogen peroxide, the by-product of methanol breakdown, is produced at a slower rate, resulting in reduced autolysis which results in vacuolar protease release into the medium.

In Figure 4.12, the methanol concentration at the end of the 24 hour period was not determined. However, in subsequent analyses on methanol depletion in shake flasks, as well as based on the YP-methanol shake flask results presented in Section 4.2.3, it was evident that no methanol was utilised by *P. pastoris*. Subsequent experimental data presented in Section 4.5, suggests the reasons why this might be so.

### 4.5.3 Effect of residual methanol on growth on cells grown in glycerol prior to methanol addition

In order to determine the effect of residual methanol on growth, cells were cultivated in chemically defined medium containing 50 g/L glycerol for 24 hours before addition of methanol at increasing concentrations as shown in Figure 4.13.Growth was monitored before and after methanol addition. Methanol addition appeared to have no significant effect on the cell morphology as observed under the microscope. Cells grown in the presence of 5 and 40 g/L methanol appeared similar in shape to those grown in the control flask with no methanol added. According to Sinha *et al.* (2004), upon growth on high concentrations of methanol, membranes become “leaky”, possibly as a result of hydrogen peroxide release, resulting in cell lysis. This results in the release of vacuolar proteases to the extracellular matrix. In order to observe trends in growth, the specific growth rates were calculated during the glycerol phase and the methanol addition phase to determine what effect (if any) methanol addition had on growth. These trends are shown in Table 4. 7 as the average growth rates achieved in each flask upon cultivation of *P. pastoris* on glycerol for 24 hours and the subsequent effect of methanol addition at the 24$^{th}$ hour. The final column of the Table 4.7 shows the residual glycerol concentration prior to the addition of methanol, determined via HPLC. The growth rates computed over the first 24 hours on glycerol were largely similar. The average growth rate obtained across the seven flasks was 0.07 ± 0.004 /h. The graphs have been normalized to control

for different starting biomass concentrations. After 24 hours' incubation on glycerol, the average biomass increase across the flasks was 4.4 ± 0.4 g/L.

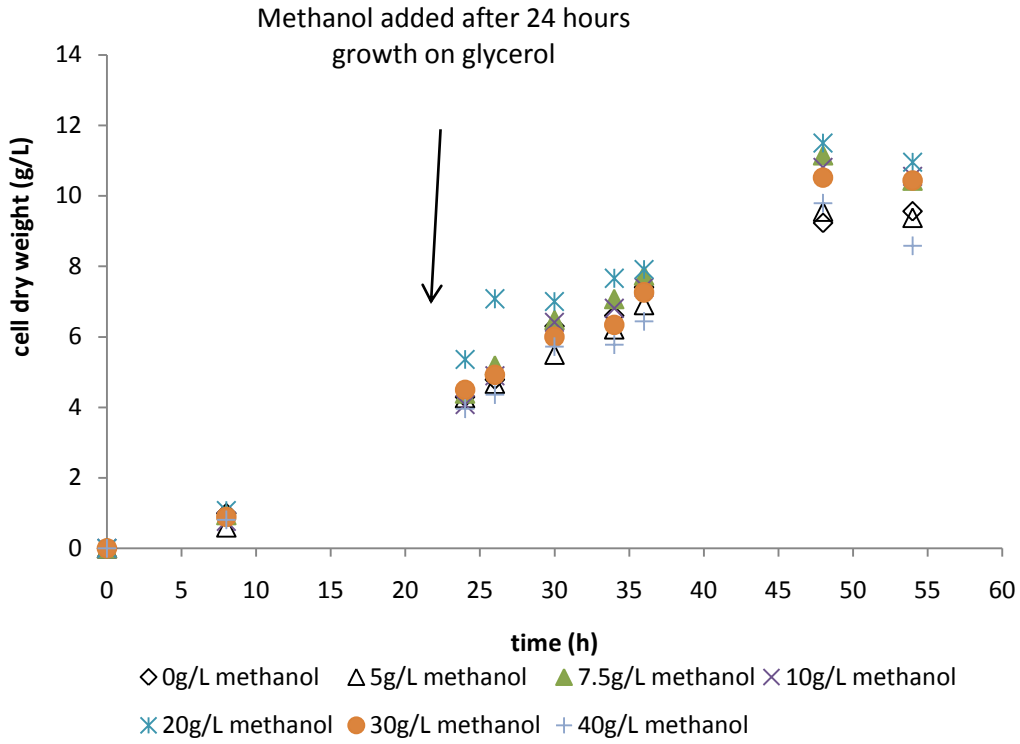


**Figure 4. 13Growth profiles of *P. pastoris* cultivated for 24 hours in chemically defined medium containing glycerol (50 g/L) followed by methanol addition at various concentrations.**

Upon 24 hours incubation (time point indicated by arrow), methanol was added at concentrations of 5, 7.5, 10, 20, 30 and 40 g/L. A flask in which no methanol was added upon 24 hours of cultivation on glycerol for 24 hours was used as a control. Results were obtained from a single experiment.

**Table 4. 7Growth rates obtained before and after methanol addition to cells grown on glycerol (50 g/L) followed by methanol addition at various concentrations**

| Flask conditions | Growth rate on glycerol, μ (/h) | Methanol concentration (g/L) | Growth rate after methanol addition, μ (/h) | Residual glycerol (g/L) at 24 hours' growth |
|---|---|---|---|---|
| **gly1(0 g/L meth)** | 0.082 | 0 | 0.025 | 14 |
| **gly2 (5 g/L meth)** | 0.073 | 5 | 0.028 | 11 |
| **gly3 (7.5 g/L meth)** | 0.08 | 7.5 | 0.028 | 19 |
| **gly4 (10 g/L meth)** | 0.074 | 10 | 0.027 | 12 |
| **gly5 (20 g/L meth)** | 0.082 | 20 | 0.022 | 11 |
| **gly6 (30 g/L meth)** | 0.076 | 30 | 0.03 | 8 |
| **gly7 (40 g/L meth)** | 0.076 | 40 | 0.033 | 13 |

Following methanol addition, the growth trajectories across the flasks appeared to be quite similar as shown in Figure 4.13. The specific growth rate values given in Table 4.7 revealed no significant differences in biomass production profiles. The average growth rate across the flasks was 0.028 /h with a deviation from this average across the flasks was 0.003 /h. This suggested that methanol addition had no impact on the growth of *P. pastoris*. This is further evident when the individual μ values on methanol were compared to the control flask where no methanol was added. In the presence of a high residual methanol concentration of 40 g/L the μ value was 0.033 /h compared to 0.025 /h in control flask whereas at the lower methanol concentration of 20 g/L the μ value was 0.022 /h. If a high residual methanol were indeed inhibitory, the growth rate values would be expected to be far lower than the growth rates obtained in the control flask. Similarly if methanol had an additive effect on growth, the μ value would be expected to be higher. Thus on the basis of these data it was difficult to conclude whether methanol was inhibitory to growth and whether such inhibition was concentration dependent. The lack of methanol uptake may have been due to the low oxygen availability and poor methanol metabolizing capabilities of the $Mut^S$ strain which lacks an alcohol oxidising gene. However, it may additionally have been due to the repression of the AOX1 promoter caused by the high residual glycerol concentrations of between 8 and 19 g/L (Table 4.7).

### 4.5.4 Effect of methanol concentration in cells grown on methanol without prior addition of glycerol

Due to the low toxicity at high methanol concentrations observed in Section 4.5.3 it was hypothesized that *P. pastoris* could withstand residual methanol concentrations of up to 40 g/L with no significant cell lysis and no inhibition of growth. Thus another experiment was conducted to determine the effect of methanol concentration on the growth of *P. pastoris* without prior addition of glycerol. Cells were cultivated in chemically defined medium containing 5, 7.5, 10, 20, 30 and 40 g/L methanol. Growth was monitored over 24 hours and the results are presented in Figure 4.14.

A lower biomass production was achieved when cells were cultivated upon methanol alone. Comparing the average final biomass achieved after 24 hours in Figure 4.14 (cultivation on methanol) to the average final biomass achieved after 24 hours cultivation on glycerol, the average biomass in methanol experiments was 3.1 ± 0.4 g/L whereas the average produced in experiments reported in Section 4.5.3 was 4.6 ± 0.8 g/L after 24 hours. The growth rates achieved, biomass produced and the methanol utilised are presented in Table 4.8. The rate of utilisation was calculated by determining the depletion of methanol due to cellular growth, accounting for the evaporation rate. The evaporation rate was computed by measuring the rate of methanol depletion in a shake

flask containing 30 g/L in chemically defined medium with no cells added (discussed in Section 4.5.4).

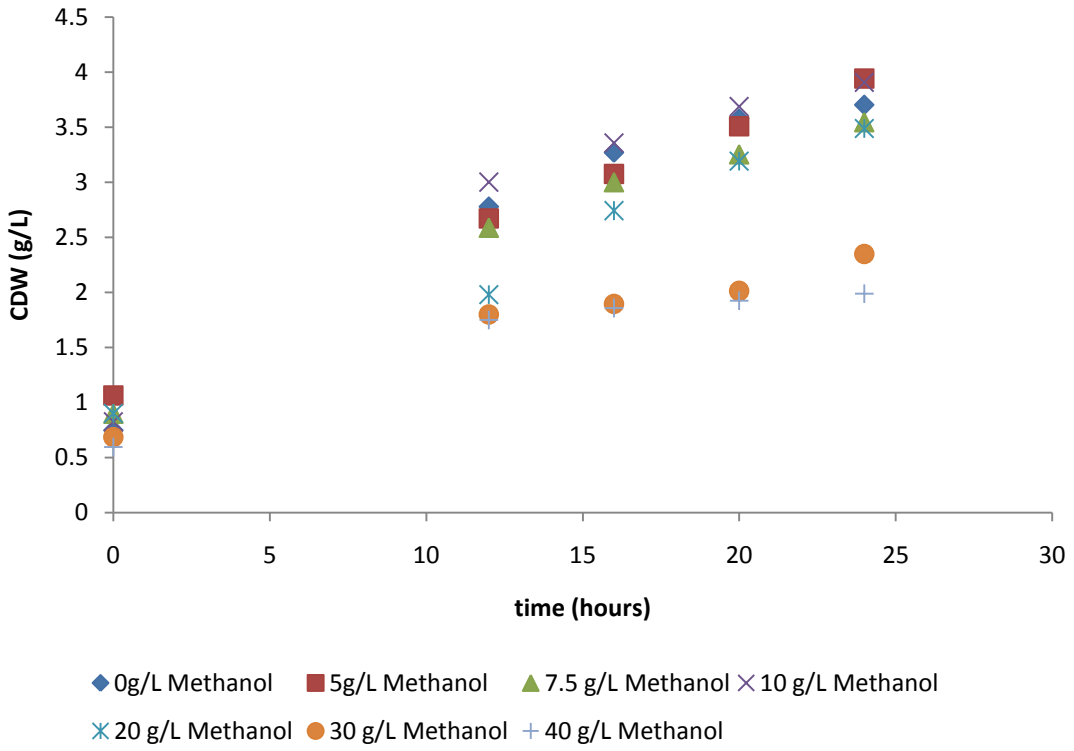


**Figure 4.14Effect of 0, 5, 7.5, 10, 20, 30 and 40 g/L methanol on the growth of *P. pastoris*in chemically defined medium.**

**Table 4. 8Growth and substrate utilisation parameters obtained when cells were cultivated in chemically defined medium containing various methanol concentrations**

| methanol added at the start of the experiment (g/L) | biomass produced after 24 hours (g/L) | specific growth rate (μ) (/h) | decrease in methanol concentration over 24 hours (g/L) | methanol utilised after accounting for evaporation rate over 24 hours (g/Lh) |
|---|---|---|---|---|
| **0** | 1.39 | 0.111 | -- | -- |
| **5** | 2.87 | 0.102 | 6 | -1 |
| **7.5** | 2.64 | 0.112 | 5.5 | -1.5 |
| **10** | 3.08 | 0.118 | 10 | 2.5 |
| **20** | 2.58 | 0.076 | 7 | 0.0 |
| **30** | 1.66 | 0.079 | 8 | 1 |
| **40** | 2.95 | 0.059 | 7 | 1 |

From the $\mu_{max}$ data shown in Table 4.8, increasing the residual methanol concentration from 0-10 g/L did not inhibit growth as evident in the comparable μ values obtained. At methanol concentrations of 20 and 30 g/L, the μvalues decreased to 0.076/h with the μ value at 40 g/L being approximately

half that obtained in the control flask with no methanol added. Statistical differences in μ values between 0-10 g/L or between 20-30 g/L could not be determined due to data being derived from a single experiment. However, based on this experiment, the fed-batch process on methanol was designed to ensure a maximum residual concentration of 10 g/L to avoid growth inhibition arising from possible accumulation of intracellular hydrogen peroxide.

### 4.5.5 Rate of methanol evaporation in shake flasks

To determine the rate of methanol evaporation a separate flask containing distilled water (no cells) and methanol at a starting concentration of 30 g/L was prepared and incubated with the experimental flasks. The rate of evaporation was found to be 0.294 g/Lh. The bar graph in Figure 4.15 compares the changes in methanol concentration over the 24 hour period observed to the expected change in methanol concentration due to evaporation measured over 12 hours. From results shown in Table 4.8 and Figure 4.15, it appears that the change in methanol concentration in flasks incubated with 5, 7.5 and 20 g/L methanol was entirely due to evaporation. The only flasks displaying apparent methanol utilisation by *P. pastoris* were the flasks containing 10 to 40 g/L methanol, where the methanol utilised by the cells (not evaporated) ranged from 0.81-2.5 g/L, representing 0.02-25% substrate utilisation. The methanol utilisation appeared to decrease at increasing residual methanol concentrations. The protease activity was additionally probed at the end of 24 hours' growth and found to be negligible with undetectable protease activity (as determined by the protease assay) in all but the flask containing 40 g/L methanol (protease activity was 0.002 units/mg, at the end of 24 hours incubation). The rate of evaporation is a function of the initial methanol concentration in the flask, as shown in Appendix G. A separate shake flask experiment to investigate the effect on the evaporation rate of four different starting methanol concentrations (10, 20, 30 and 40 g/L) over 12 hours found the average evaporation rate of evaporation to be 0.29 ± 0.04 g/Lh. The data is presented in Appendix G. Thus the effect of the starting methanol concentration on the evaporation rate was shown to lie within the experimental error of the system.

The results support the conclusion that a residual methanol concentration of 10 g/L and below was non-inhibiting to growth of *P. pastoris* in chemically defined medium. However, these results do not suggest that *P. pastoris* was able to grow on methanol. The comparable growth rate achieved in the control flask (no methanol added) compared to growth on 5-10 g/L methanol indicated that a similar growth trajectory and final biomass concentration could result in the absence of methanol. The low methanol consumption and simultaneous biomass production led to the speculation that biomass production was likely due to the carbon present in yeast extract and peptone carried over from the

inoculum culture. Cells were pre-cultured on a yeast-peptone medium (1% yeast extract; 2% peptone) for 24 hours before being inoculated into the chemically defined medium containing various methanol concentrations. The size of the inoculum was 10% (v/v). Thus it is posited that the growth observed was due to utilisation of carbon present in yeast-extract and peptone. These results indicated a threshold concentration of 10 g/L methanol beyond which growth inhibition from methanol addition results. Furthermore, evidence of methanol-dependent protease release was limited, similar to that observed in the complex medium.

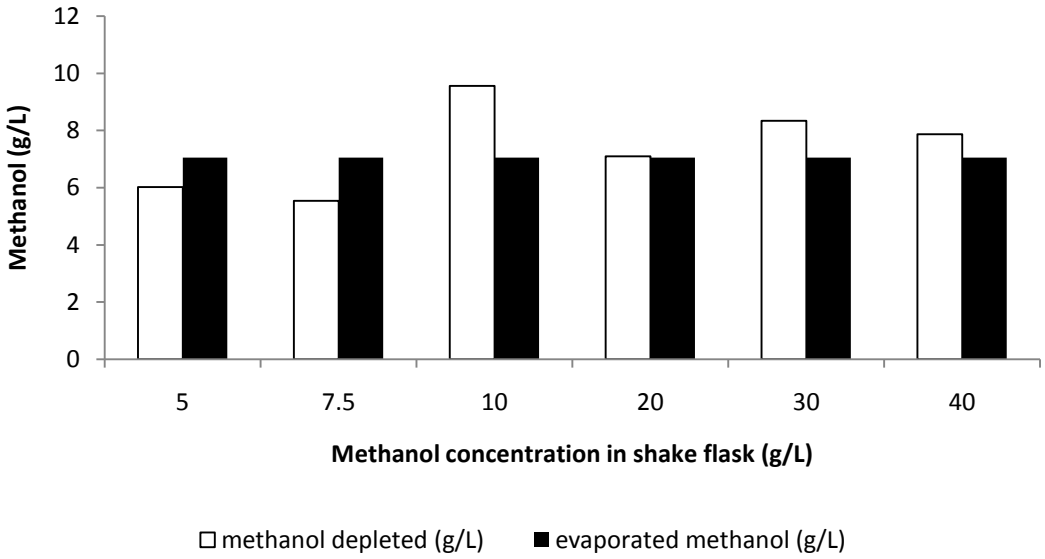


**Figure 4. 15Comparison of observed methanol depletion over 24 hours to the methanol depleted due to evaporation.**
A separate flask was incubated in identical conditions to experimental flasks, containing methanol at 30 g/L in distilled water. The change in methanol concentration in this flask was used to calculate the methanol evaporation rate. Thus the graph compares the expected depletion of methanol due to evaporation ("evaporated methanol") to the change in methanol concentration observed in each flask over the course of the experiment ("methanol depleted").

The response of the $Mut^S$ strain used in this study differed from that of the wild-type ($Mut^+$) strain. In this strain, methanol is hydrolysed rapidly within membrane-bound organelles known as peroxisomes. The excessive accumulation of methanol hydrolysis byproducts, i.e. formaldehyde and hydrogen peroxide, is inhibitory to growth. Therefore a residual methanol concentration of approximately 3.65 g/L is recommended (Cregg, 2004). However, as previously noted, the lower enzyme concentration in the $Mut^S$ strain used in this study resulted in slower metabolism of methanol, thereby resulting in slower accumulation of the toxic by-products. The effect of methanol concentration on the production of insulin precursor and the time-dependent effect on protease release or cell lysis upon prolonged periods of exposure to methanol were not investigated in shake flask experiments.

The presence of glycerol may prevent methanol uptake due to repression of the AOX promoters as suggested by data presented in Section 4.5.3. The shake flask studies indicated that a residual methanol concentration of up to 10 g/L could be maintained without inhibiting growth or releasing proteases. Thus the fed-batch process was designed to ensure that no glycerol was present during the methanol induction phase.

## 4.6 Two-stage fed-batch system for biomass production and induction of recombinant insulin precursor production

### 4.6.1 Introduction

As mentioned in Section 2.12, available literature on process development of *P. pastoris* for insulin production is limited. For instance, one group has focused on optimising the methanol induction phase following a single batch phase (Gurramkonda *et al.*2010). Another attempted to determine the best pH and temperature conditions (Pais-Chanfrau *et al.*2004). A third group investigated the effect of insulin precursor gene copy number on insulin production (Mansur *et al.*2005). This study sought to develop an approach for large-scale production of insulin in *P. pastoris*by optimising the overall bioprocess. By using kinetic parameters derived from batch experiments to design a second exponential glycerol feed, a fed-batch process was designed to enhance biomass production prior to insulin production. From Figures 4.9 and 4.10 (Section 4.3.1), it was evident that yeast extract and peptone were utilised in preference to glycerol with glycerol utilisation increasing on their depletion. To avoid this preferential use of alternative carbon sources over glycerol a chemically defined medium containing glycerol was used, allowing respiratory growth with no observable ethanol production.

Oxygen was supplied to ensure a minimum concentration of 20-30 % dissolved oxygen which amounted to 1.8-2.4 mg/L. The value for distilled water at 30$^{o}$C and 1 atm was used. . The major considerations for the methanol induction phase were ensuring minimal protease release and the use of a methanol concentration that was not inhibitory to *P. pastoris*. Protease release in the shake flask experiments conducted was significantly lower than the observed protease activity in the literature  a maximum residual protease activity of 0.076 units/mg was observed vs. 210 units/mg reported by Sinha *et al.* (2004). Further, methanol appeared growth inhibitory above an initial methanol concentration of 10 g/L in batch culture (Section 4.5.4). Thus to ensure optimal methanol induction conditions, a residual methanol concentration below 10 g/L during the fed-batch induction phase was desired. Two initial fed-batch experiments were performed to optimise the glycerol and methanol fed-batch conditions. The methanol utilisation rate, determined iteratively, formed the basis for the methanol induction phase conditions employed for the optimised fed-batch

experiments. Initially the model used for the fed-batch addition of methanol is presented (Section 4.7) followed by the preliminary fed-batch runs. Finally, data from the optimised fed-batch runs are presented.

### 4.7 Fed-Batch model development

The overall aim of this project was to optimise a fed-batch insulin precursor production process in *P. pastoris* in which the first fed-batch stage is operated as a "μ-stat" such that biomass was produced at a pre-determined growth rate by limiting the availability of a single nutrient (the carbon source). This approach makes it possible to design a process producing high cell concentration and product concentration through the controlled addition of substrate. This prevents undesirable side-effects like catabolite repression, substrate inhibition and overflow metabolism leading to ethanol production arising from over-feeding or underfeeding. By manipulating a specific process parameter, such as cell concentration, product formation or productivity, a fed-batch process can be designed.

Thus by developing a model to describe the behaviour of *P. pastoris* during growth and product formation stages, the overall process could be optimised. In this dissertation, the primary focus is on attaining a high cell density cell culture with secondary focus on the recombinant insulin precursor product.

The model developed describes a process that entails a two-stage growth process on carbon (glycerol): initial batch growth followed by a fed-batch supply of the same carbon source to boost biomass. The final fed-batch stage involved supply of methanol to induce gene expression. The model used to determine the feed profile and expected biomass growth trajectory regarded cells as homogeneous with no plasmid instability, because the vector was integrated into the yeast chromosome.

The fed-batch system has a variable volume, whereby materials can enter but cannot exit. The volume of the reactor, V(t) is a function of time (t), and is defined by the substrate flow rate F(t). The concentration of the two carbon substrates, glycerol (G) and methanol (M), in the vessel is affected by the flow rate, their feed concentration and the rate of their consumption, the latter being determined by metabolic activity and cell concentration, X. The resultant cell concentration is dependent on the total amount of substrate available (i.e. added to reactor) up to any given time and the dilution effect of the increasing volume. The rate of product formation which influences product concentration, P, is a function of the specific product formation rate and the cell concentration ($q_pX$). The reactor is assumed to be well-mixed with no regions of non-homogeneity.

Initially, cells were grown in batch mode during which an initial glycerol charge was provided, allowing cells to grow until the carbon source was exhausted. The system volume was unchanged, product formation was negligible due to repression of the promoter and growth was assumed to follow Monod-type kinetics. Equations 3.1 and 3.2 describe the batch mode of growth.

$$X = X_0 e^{\mu t} \qquad \text{Equation 4.1}$$

$$\frac{dG}{dt} = -\frac{1}{Y_{x/G}} \mu X \qquad \text{Equation 4.2}$$

where μ is the specific growth rate, $Y_{X/G}$ is the yield of biomass on the carbon source.

$$X = X_0 + Y_{X/G}(G_0 - G) \qquad \text{Equation 4.3}$$

Equation 4.4 describes biomass production throughout the batch phase. Upon exhaustion of the initial carbon substrate, glycerol or glucose was fed to the reactor to boost further cell growth during the fed-batch phase. The biomass balance as the cell concentration increases is given by

$$XV = X_T = X_0 V_0 + Y_{X/G}\, F(t)(G_f - G) \qquad \text{Equation 4.4}$$

where $G_f$ is the carbon concentration in the feed.

The change in volume of the reactor at any given point *t* during the fed-batch process is given by Equation 4.5.

$$V(t) = V(t_0) + \int_{t_0}^{t} F(t)dt \qquad \text{Equation 4.5}$$

where $V(t_0)$ is the volume of the reactor at the end of the batch phase and at the beginning of the fed-batch cultivation period. The increase in volume is dependent on the carbon (glucose or glycerol) concentration in the feed and the rate at which it is fed.

The change in the biomass concentration during the fed-batch phase is given by Equation 4.6

$$X(t) = X(t_0)V(t_0) + Y_{X/G} \int_{t_0}^{t} F(t)dt \qquad \text{Equation 4.6}$$

The product concentration P is predicted as a function of biomass and volume change during the fed-batch cultivation period, by Equation 4.7 where $q_p$ is the specific rate of product formation.

$$P(t) = \frac{q_p \cdot \int_{t_0}^{t} X(t).V(t)dt}{V(t)} \qquad \text{Equation 4.7}$$

For fed-batch cultivation, a constant specific growth rate (μ) can be maintained by feeding the carbon substrate in a controlled exponential manner. For a fed-batch system based on a single substrate S, where a quasi steady state exists for the residual substrate, the specific growth rate is given by Equation 4.8

$$\mu(t) = \frac{Y_{X/G}.G_0.F(t)}{X(t).V(t)} \qquad \text{Equation 4.8}$$

To maintain the specific growth rate at a constant rate, the cell balance is integrated to determine the required flow rate by Equation 4.9

$$F(t) = \frac{\mu.X.V(t)}{G_0.Y_{X/G}} \qquad \text{Equation 4.9}$$

For the fed-batch process initiated after an initial batch phase, the flow rate required to maintain a specific growth rate is given by equation 4.10. *X* is the biomass concentration at the start of the fed-batch phase, $t_0$. *V* is the bioreactor volume at the start of the fed-batch phase

$$F(t) = \frac{\mu.X(t_0).V(t_0).e^{\mu(t-t_0)}}{Y_{x/s}.G_0} \qquad \text{Equation 4.10}$$

By determining the input parameters X ($t_0$), V ($t_0$), $Y_{X/S}$ at the end of the batch phase and by feeding glycerol of a pre-determined concentration, $G_0$ to maintain a preset growth rate (=μ) the flow rate for any time point ($t_0$ = 0 h) throughout the fed-batch phase could be determined by Equation 3.11. Based on constant measurements of the feed bottle as well as the calculated feed concentration, the amount of substrate to be added could be determined gravimetrically. Further, constant measurement of substrate concentrations during the fed-batch phase confirmed that no glycerol was accumulating in the reactor.

### 4.8 Preliminary fed-batch experiments

Fed-batch process setup and operation is described in Section 3.7. The initial batch stage used chemically defined medium containing 50 g/L glycerol. Growth continued in batch phase until depletion of the glycerol charge, associated with a sharp increase in dissolved oxygen concentration (not shown). This signalled transition to the fed-batch addition of glycerol using a 200 g/L glycerol feed described in Section 3.7. The exponential glycerol feed was continued until the reactor volume reached 4 L. On transition to a methanol phase, a pulse of 20 g of methanol was first added to the reactor to induce its utilisation; the dissolved oxygen concentration decreased as the cells acclimatised to methanol. In general, the purpose of the methanol pulse was to 'prime' the AOX enzyme system to allow for the metabolism of methanol during induction. Typically the dissolved

oxygen would drop upon addition of this pulse and remain low for 4-5 hours. Upon depletion of the initial methanol pulse, indicated by an increase in the dissolved oxygen levels, the induction phase was initiated. Two preliminary runs were operated in order to optimise each phase of the overall fed-batch process.

Results of the first fed-batch run to optimise the glycerol fed-batch phase are shown in Figure 4.17. Cells were initially grown in batch phase, with a $\mu_{max}$ of 0.19 /h. After the 22$^{nd}$ hour of growth the glycerol was depleted (indicated by a sharp increase in the dissolved oxygen reading) and the biomass concentration reached 22.9 g/L. Glycerol fed to maintain the pre-determined growth rate was 0.15 /h and the feed rate was monitored gravimetrically. The residual substrate concentration was determined throughout the fed-batch process and the flow rate adjusted to ensure that the concentration of residual glycerol in the reactor was below the detectable limit. Cells grew exponentially over seven hours of fed-batch cultivation. The biomass concentration increased from 25.3 g/L at the start of fed-batch cultivation to 35.2 g/L in the third hour of fed-batch cultivation. However, at this point, the residual glycerol concentration was 12 g/L, rising to 14 g/L four hours into exponential growth. This indicated glycerol delivery at a faster rate than used. The glycerol feed rate was reduced, subsequently reducing the residual glycerol concentration to 1.39 g/L in the fifth hour of fed-batch operation and an undetectable concentration by the sixth hour. During this period, the dissolved oxygen level was maintained at 20-30% saturation by mixing air with pure oxygen to ensure that dissolved oxygen was above the critical concentration required for growth. Plotting the logarithm of total cell dry weight $X_T$ ($X_T$= X(t) (g/L) x V(t) (L)) against time showed that the gradient of the graph obtained (shown in Figure 4.15), i.e. the specific growth rate remained at 0.15 /h, matching the predetermined rate based on the equation 4.10.

Biomass production in the batch phase of the second preliminary run, given in Figure 4.18, showed a similar trajectory to the first run, with a maximum specific growth rate obtained during the batch phase of 0.21 /h and a final biomass at the end of batch phase of 27 g/L after the 22$^{nd}$ hour. During fed-batch growth on glycerol, the preset growth rate was maintained at 0.21 /h in order to boost biomass production. Biomass increased from 27 g/L at the start of the fed-batch stage to 52 g/L at the end of glycerol fed-batch growth after the 26$^{th}$ hour. Glycerol was initially overfed to the reactor in the first hour, reaching a concentration of 12.2 g/L. However, upon adjustment of the flow rate, no further accumulation was observed. Generally, to prevent overfeeding, the rate at which glycerol was fed ought to be lower than that required to maintain the $\mu_{max}$ value. In this experiment, the preset growth rate was equal to the maximum specific growth rate. However, no acetate or ethanol accumulation was observed. The average glycerol feed rate over the fed-batch growth on glycerol

was 51 g/h. In order to acclimatise the cells to methanol at the end of glycerol feeding, a 20 g pulse of methanol was added to the reactor leading to a decrease in the dissolved oxygen level as the methanol pulse was depleted. The reactor was left overnight with glycerol fed at the lowest pump speed in order to prevent cell starvation. This resulted in an increase in the biomass from 62 to 68.7 g/L observed between the 26$^{th}$hour and the 40$^{th}$ hour at a growth rate of 0.08 /h. The methanol fed-batch phase was initiated at t = 40 h. Initially, the pump rate was adjusted to provide 10 g/Lh. However, by the 3$^{rd}$ hour of feeding, it was evident that an excessive quantity of methanol had been fed (residual concentration was 21 g/L methanol). This further increased to 28 g/L in the 4$^{th}$ hour of methanol fed-batch phase. At this stage the feed was discontinued completely and the rate of methanol depletion was measured over the next 4 hours. These results are shown in Figure 4.18. The rate of methanol depletion observed was 7.25 g/Lh (Figure 4.19). This rate however did not account for the methanol depleted due to evaporation. The methanol evaporation rate was experimentally determined to be 0.49 g/Lh in bioreactor conditions (see Appendix G for details). Thus the methanol depletion that was attributable to *P. pastoris* was 6.8 g/Lh. In subsequent fed-batch experiments, the feed rate was therefore set to ensure that the maximum rate of methanol addition did not exceed 7g/Lh. Methanol was fed at a constant rate unlike glycerol prior to methanol addition. Methanol was added at a non-inhibiting rate in order to induce gene expression.

Following the two preliminary optimisation runs, three independent fed-batch runs were conducted to assess this approach to biomass production and gene induction by methanol. Of specific interest was whether additional biomass production occurred during the methanol induction phase. Dissolved oxygen was maintained at 30% of air saturation by mixing pure oxygen with air and by increasing the agitation from 600 rpm (during batch) to 800 rpm for the fed-batch phase.

### 4.8.1 Optimised fed-batch runs

Figure 4.20 shows the results of the first fed-batch run performed using the optimised fed-batch conditions. At the end of the batch phase at t=24 h, the biomass concentration was 24 g/L with the $\mu_{max}$ during batch cultivation being 0.19 /h. The glycerol feed was initiated at the 24$^{th}$hourto ensure a predetermined specific growth rate of 0.17 /h. The biomass concentration increased from 24 g/L at t=24 h to 57 g/L at the end of the glycerol fed-batch cultivation at 30 hours, representing a 148 g increase in total biomass. The biomass increased from 57 g/L to 78 g/L between the 30$^{th}$ and the 38$^{th}$ hour, due to slow glycerol addition (0.04 /h) to ensure that *P. pastoris* cells were not starved. In addition 20 g of methanol was pulsed as for the first fed-batch at 38 h.

At the 38th hour, the residual methanol and glycerol concentration was below the detection limit. The methanol induction phase was initiated by fed-batch addition of methanol at an average methanol feed rate of 3.8 g/Lh and the increase in biomass production occurred at a rate of 0.04 /h. The induction phase from the 38th to the 82nd hour was characterized by a stable cell concentration of 74 g/L with a slight deviation of 1.6 g/L while the reactor volume increased from 4 to 5 L. During this period the residual methanol concentration was undetectable by HPLC (detection limit 0.5 g/L).

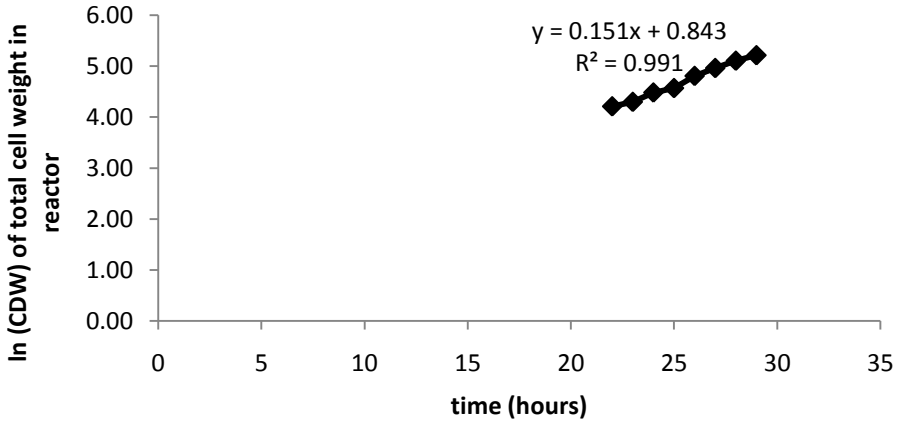


**Figure 4. 16The logarithm of cell dry weight plotted against time to confirmthe pre-determined feed rate to be 0.15 /h**

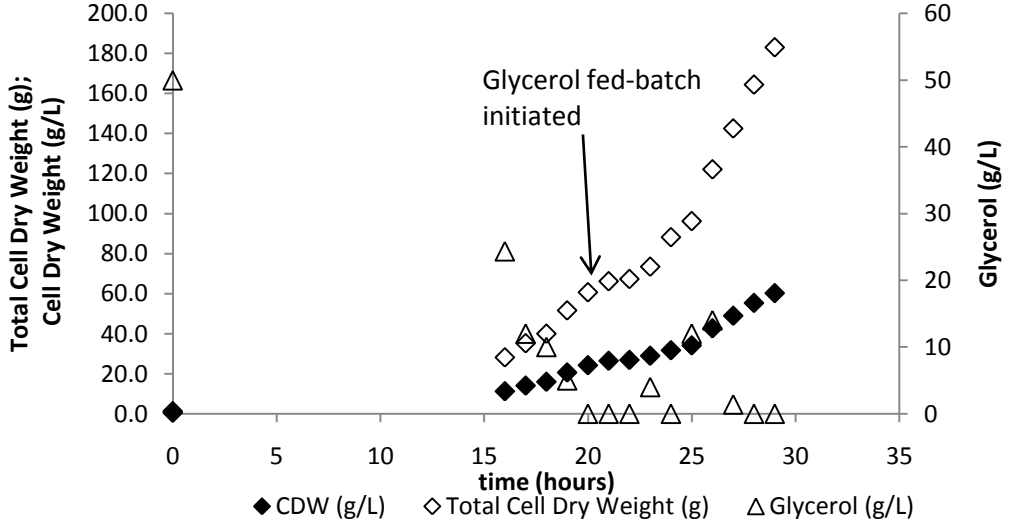


**Figure 4. 17Graph indicating biomass production cell and substrate concentration over time in the preliminary fed-batch optimisationexperiment employing glycerol for fed-batch growth**.
Cells were grown in chemically defined medium (reactor volume 2.5 L) containing 50 g/L glycerol in batch phase until glycerol was exhausted (at t= 22 h). Fed-batch cultivation on glycerol was initiated at t=22 h and continued until t= 29 h. Glycerol was fed at an average rate of 66 g/h during fed-batch. No methanol induction was initiated in this run. The biomass, glycerol and methanol concentrations are shown. A maximum biomass concentration of approximately 60 g/L was obtained, representing total biomass production of 116 g during fed-batch on glycerol. Glycerol was monitored regularly during fed-batch in order to maintain its concentration below detection levels during fed batch growth.

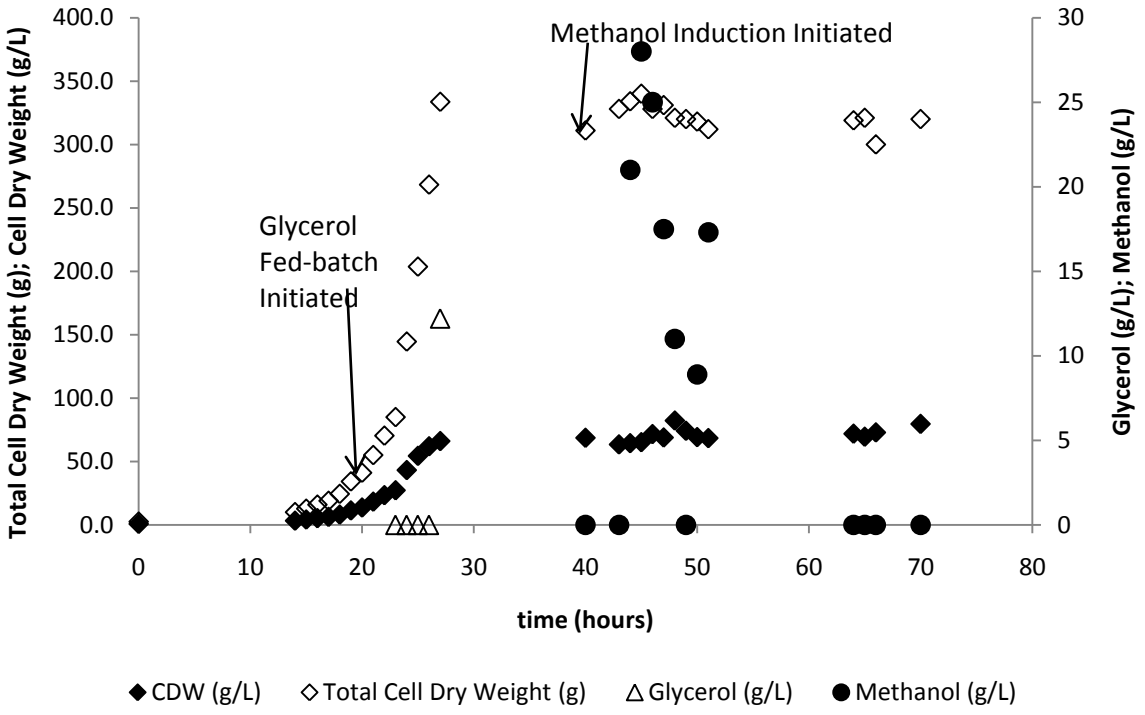


**Figure 4. 18Graph indicating total biomass production, cell and substrate concentration over time in the second preliminary fed-batch optimisationrun where glycerol and methanol fed-batch phases were employed for growth and induction respectively.**

Cells were grown in chemically defined medium containing 50 g/L glycerol in batch phase until glycerol was exhausted (at t= 22 h). Fed-batch cultivation on glycerol was initiated at t=22 h at a preset feed rate of 0.21 /h and continued until t= 29 h. The average glycerol feed rate was approximately 47 g/h. A pulse of methanol (5 g/L) was added to the reactor at the 29$^{th}$ hour and the reactor left overnight. The methanol fed-batch was initiated at t = 40 h. The total biomass and biomass concentration (g/L), glycerol and methanol (•) concentrations (g/L) are shown in the graph.

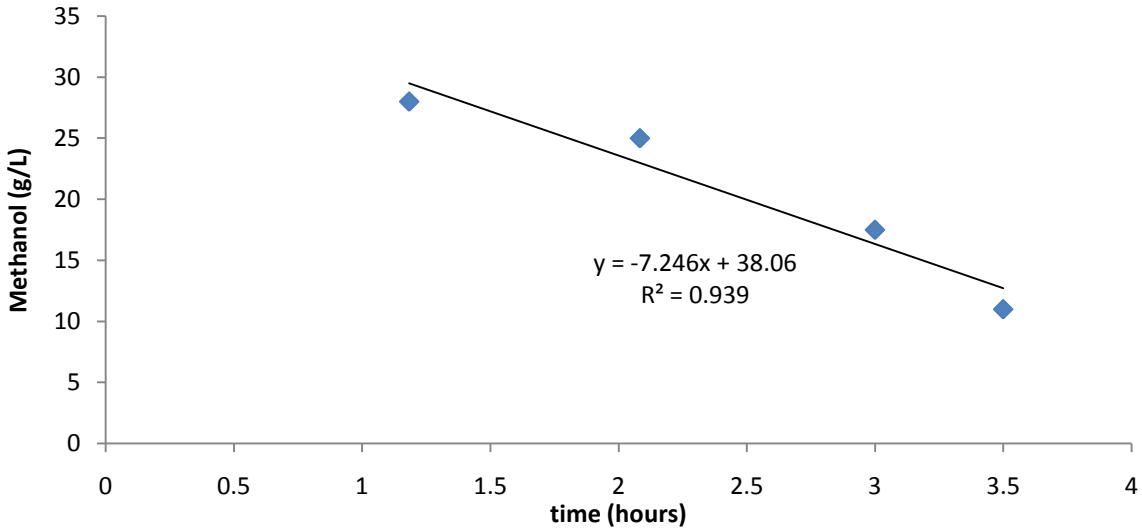


**Figure 4. 19Decrease in methanol concentration over time.**

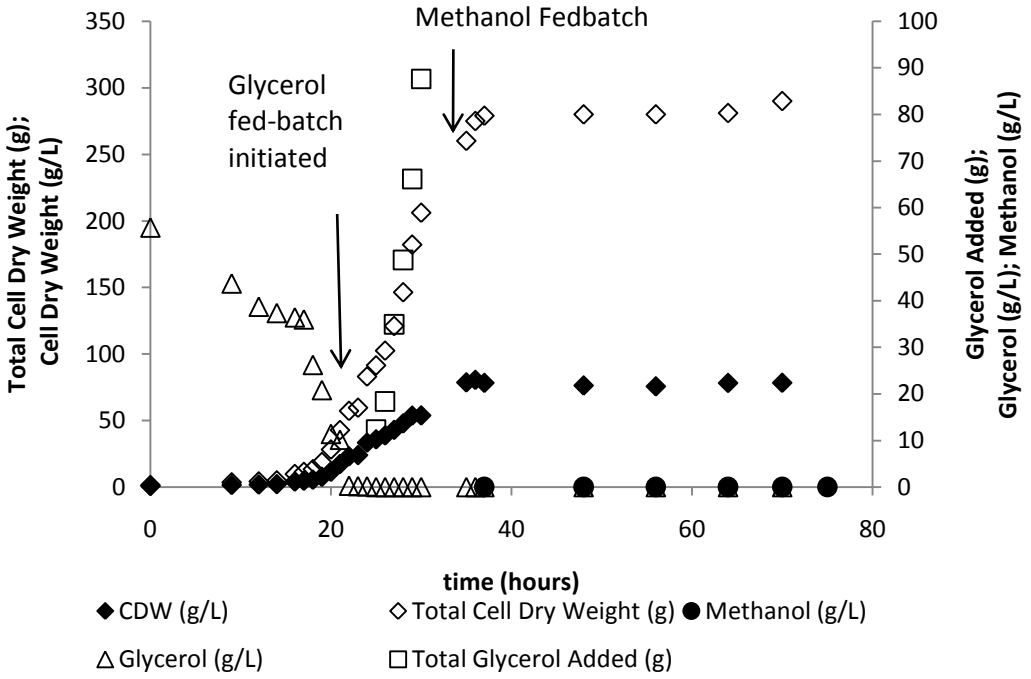


**Figure 4. 20Graph indicating total biomass production and cell concentration over time in the third run, the first optimised fed-batch run to cultivate *P. pastoris* and induce insulin production**.
Cells were grown in chemically defined medium containing 50 g/L glycerol in batch phase until glycerol was exhausted (at t= 24). The final batch volume at the end of run 3 was 2.5 L. Fed-batch cultivation on glycerol was initiated at t=24 h at a preset feed rate of 0.17 /h and continued until t= 30 h. Glycerol was fed at an average rate of 45 g/h. A pulse of methanol (5 g/L) was added to the reactor at the 30$^{th}$ hour and the reactor left overnight. Additionally a glycerol feed was provided at 0.04 /h to prevent cell starvation. The methanol fed-batch was initiated at t=38 h. Methanol was fed at 3.8 g/Lh. The biomass (◊), glycerol (Δ) and methanol (•) concentration profiles (in g/L) are shown. No glycerol and methanol accumulation occurred during the two fed-batch stages.

Figure 4.21 shows the results obtained from the second optimised run. To assess the reproducibility of this approach, Run 4 was a repeat of Run 3, with the same fed-batch parameters and preset growth rates. The maximum specific growth rate during the batch phase was 0.2 /h and the biomass achieved at the end of the batch phase was approximately 30 g/L.As in Run 3, exponential growth at a specific growth rate of 0.17 /h was maintained during fed-batch cultivation on glycerol. At the end of batch phase, 20 g methanol was added to the reactor as a pulse and incubated overnight. No further addition of glycerol occurred. This initial methanol quantity was fully depleted by the start of the methanol induction phase at the 42$^{nd}$ hour. The biomass concentration remained relatively stable at 78 ± 5.4 g/L during the induction phase but due to the change in volume, the total biomass increased from 230 g to 282 g at the end of the methanol fed-batch phase.

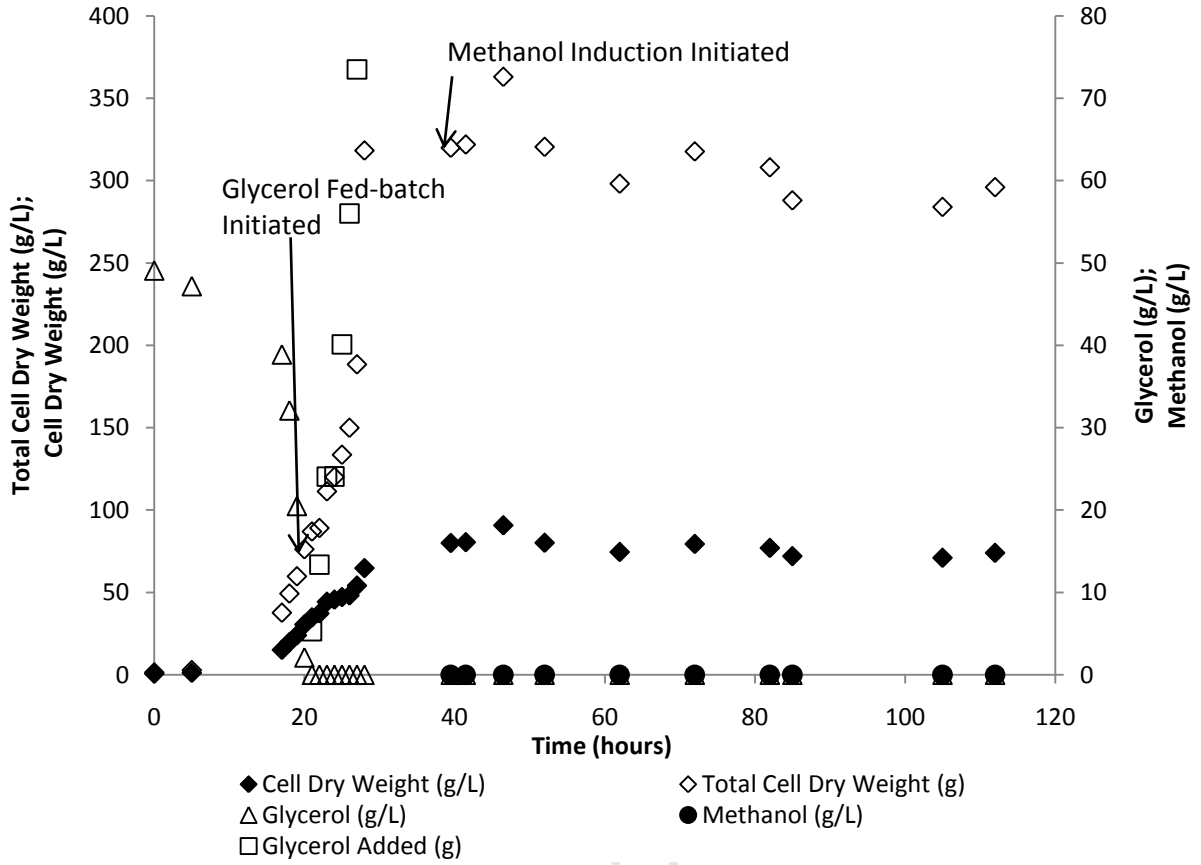


**Figure 4. 21indicating total biomass production and cell concentration over time in the fourth run, second optimised fed-batch run to cultivate *P pastoris* and induce insulin production.**

Cells were grown in chemically defined medium containing 50 g/L glycerol in batch phase until glycerol was exhausted (at t= 20 h) at a batch volume of 2.5 L. Fed-batch cultivation on glycerol was initiated at t= 21 h at a preset feed rate of 0.17 /h and continued until t = 28 h. Glycerol was fed at an average rate of 45 g/h. A pulse of methanol (5 g/L) was added to the reactor at the 30$^{th}$ hour and the reactor left overnight. The methanol fed-batch was initiated at t=38 h. Methanol was fed at the newly determined methanol utilisation rate of 2.8 g/Lh.The biomass, glycerol and methanol concentrations (in g/L) and total glycerol added and total biomass formed (g) are shown in the graph. No glycerol and methanol accumulation occurred during the two fed-batch stages.

The results of the third optimised fed-batch experiment are shown in Figure 4.22. Batch cultivation in chemically defined medium containing glycerol (50 g/L) was as described in the previous two optimised runs. The maximum specific growth rate achieved was 0.23 /h and a maximum biomass concentration of 30.5 g/L were obtained. Glycerol was exhausted at the 20$^{th}$ hour and fed-batch was initiated at the 21$^{st}$hour. The preset growth rate was set to 0.21 /h for 6 hours. At t = 30 h, a 20 g pulse of methanol was added to the reactor and the reactor left for 14 hours until the 44$^{th}$ hour with slow addition of glycerol, before initiating the induction phase. This led to growth at 0.006 /h between the time points of 30 h and 44 h with a biomass concentration increasing from approximately 60 g/L to approximately 80 g/L (with biomass increase from 166 to 208 g overnight . The methanol induction phase was initiated at the 44$^{th}$ hour with methanol added to the reactor at a flow rate of 2.9 g/Lh. Glycerol and methanol concentrations remained below the detection limit

throughout the glycerol fed-batch phase and the methanol induction phase. Generally, the average biomass concentration remains stable during the fed-batch phase.

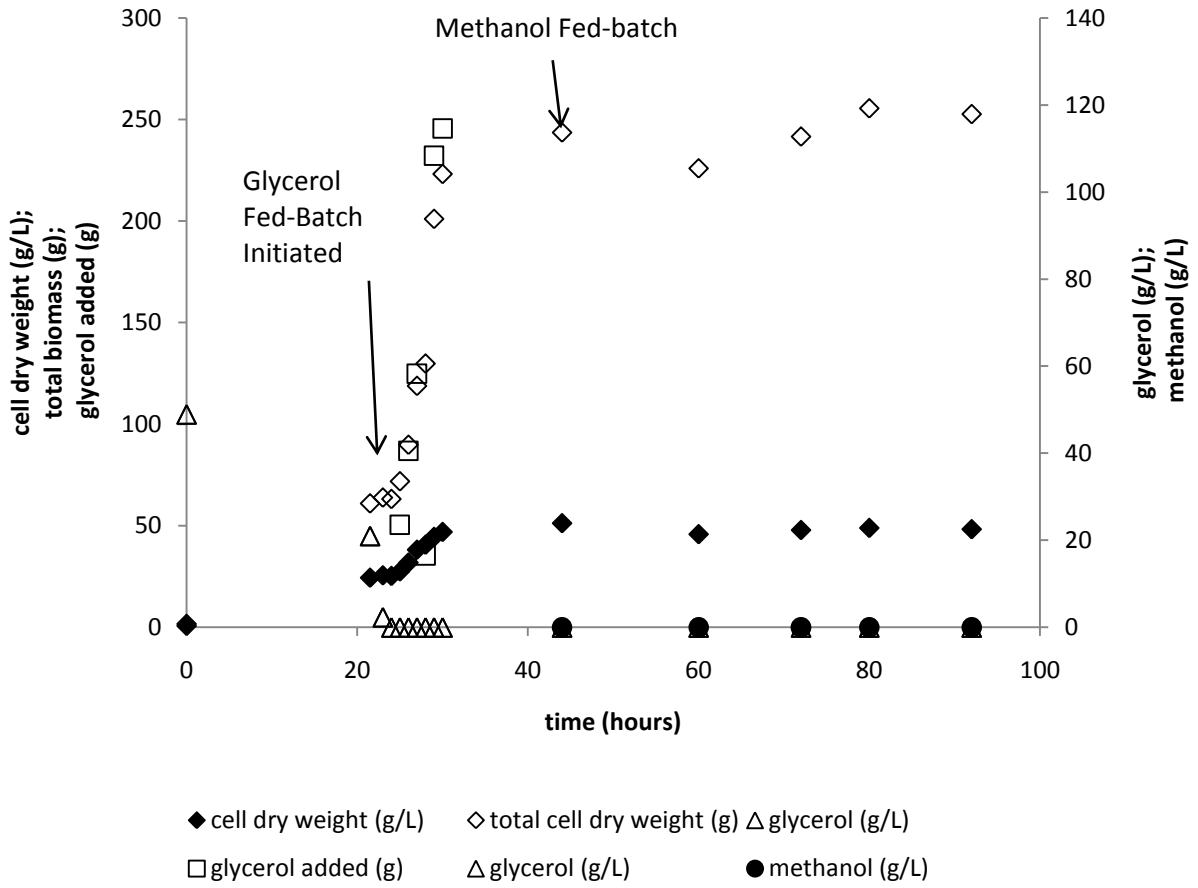


**Figure 4. 22 Run 5, third optimised fed-batch run to cultivate *P. pastoris* and induce insulin production**.
Cells were grown in chemically defined medium containing 50 g/L glycerol in batch phase until glycerol was exhausted (at t= 24 h). Fed-batch cultivation on glycerol was initiated at t=25 h at a preset feed rate of 0.21 /h and continued until t= 30 h. Glycerol was fed at an average rate of 55 g/h. A pulse of methanol (5 g/L) was added to the reactor at the 30th hour and the reactor left overnight. The methanol fed-batch was initiated at t=44 h. Methanol was fed at the newly determined methanol utilisation rate of 2.9 g/Lh. The biomass, glycerol and methanol concentrations (in g/L) and the total glycerol added and biomass formed (g) are shown in the graph. No glycerol and methanol accumulation occurred during the twofed-batch stages

**Table 4.9 Summary of kinetic parameters obtained during optimised fed-batch runs.**

| | Fed-batch 3 | Fed-batch 4 | Fed-batch 5 |
|---|---|---|---|
| **Batch on glycerol** | | | |
| **Initial glycerol conc (g/L)[1]** | 55 | 53 | 49 |
| **$\mu_{max}$ (/h)[2]** | 0.19 | 0.20 | 0.23 |
| **$Y_{x/s}$ (g/g)[3]** | 0.43 | 0.49 | 0.51 |
| **$X_{max}$ (g/L)[4]** | 24.07 | 26.52 | 25.29 |
| **Fed- batch on glycerol** | | | |
| **µ (/h) set[5]** | 0.17 | 0.17 | 0.21 |
| **µ achieved?[6]** | 0.16 | yes | yes |
| **Increase in biomass (g)[7]** | 148 | 151.7 | 110 |
| **Vol at start (L)[8]** | 2.5 | 2.5 | 2.5 |
| **Gly feed conc (g/L)[9]** | 200 | 200 | 200 |
| **Vol added (L)[10]** | 1.59 | 1.59 | 1.39 |
| **Vol at end (l)[11]** | 4.09 | 4.09 | 3.89 |
| **$Y_{x/glycerol}$ (g/g)[12]** | 0.47 | 0.48 | 0.41 |
| **duration of fedbatch(h)[13]** | 6 | 7 | 5 |
| **Fedbatch on methanol** | | | |
| **Feed conc (g/L)[14]** | 200 | 200 | 200 |
| **Methanol feed rate (g/L/h)[15]** | 3.8 | 2.8 | 2.94 |
| **Duration (h)[16]** | 52 | 68 | 62 |
| **µ (methanol) (/h)[17]** | 0.006 | 0.002 | 0.005 |
| **$Y_{x/methanol}$ (g/g)[18]** | 0.3 | 0.3 | 0.3 |
| **Change in biomass (g)[19]** | 72.2 | 56 | 60 |

[1] the measured glycerol concentration at the start of the batch phase; [2] maximum specific growth rate computed by plotting ln(biomass) vs. time and calculating the gradient over the linear portion of the graph; [3] change in biomass over entire batch phase divided by total change in substrate concentration; [4] highest biomass concentration produced in batch phase; [5]the programmed glycerol feed rate for the fed-batch phase on glycerol; [6] was the preset growth rate achieved?; [7]change in total biomass produced obtained from product of cell concentration (g/L) and volume of reactor (L); [8] volume of the reactor at the start of the fed-batch phase; [9] glycerol feed concentration for the batch phase (g/L); [10] calculated by dividing the mass of feed added (as measured during fed-batch phase) by the density of the feed; [11]sum of volume added and the volume of the reactor during batch phase; [12] total biomass formed divided by total glycerol added (conc of feed x volume added); [13] Measured time from the start of the fed-batch phase until glycerol feed was halted; [14] concentration of methanol feed in feed bottle for methanol induction phase; [15] average rate of methanol addition, calculated by dividing total methanol added by the overall duration of the induction phase. This concentration was not constant and was adjusted to ensure that no methanol accumulated in the reactor; [16] measured time from start of methanol constant feed (not the initial acclimatisation period) till the end of the experiment (typically upon exhaustion of methanol feed); [17] growth rate on methanol computed by plotting ln (total biomass during methanol phase) against time and taking the gradient over the entire period; [18]total biomass produced during methanol phase (concentration in g/L multiplied by volume) divided by total methanol added (g); [19] biomass concentration at the end of experiment- biomass concentration at the start of induction phase (g)

Table 4.9 presents a summary of the kinetic parameters obtained in the three optimised fed-batch runs. The batch conditions across the three experiments were reproducible. The average starting glycerol concentration for the batch phase was 52.3 ± 3.0 g/L glycerol; cells grew at an average $\mu_{max}$ of 0.2 ± 0.01 /h producing a final biomass concentration of 25.3 ± 1.2 g/L with an average yield on substrate of 0.48 ± 0.04g/g. The duration of batch phase was 23.7 ± 0.7 hours.

In each of the fed-batch runs, the reactor volume at the start of the fed-batch process was 2.5 L. In run 3, the attained exponential feed rate (0.16 /h) was slightly below the preset growth rate (0.17 /h) possibly due to errors in the pump calibration and setup as well as in biomass measurements. The glycerol fed-batch runs 4 and 5,however,attained the predetermined exponential growth rates. In determining the preset feed rates, the major considerations included maximizing biomass production without over-feeding substrate. For this reason, the feed rate was calculated to give a specific growth rate below the $\mu_{max}$ value observed during batch growth. The considerations involved in determining the reactor volumes are discussed in Section 3.7. The biomass increases obtained in runs 3 and 4 were much higher (148 and 152 g) than the increase in biomass attained in run 5 (110 g). The biomass yields were similarly higher in runs 3 and 4 than in 5 (0.47-0.48 in runs 3-4 compared to 0.41 in run 5). The reason for the higher yields as well as higher biomass production in runs 3 and 4 (compared to run 5) is possibly due to the exponential feeding rate of glycerol being sufficiently lower than the $\mu_{max,}$ thus preventing overflow metabolism. Generally, overflow metabolism results when the substrate uptake rate exceeds the rate at which the substrate can be respired. The carbon flux becomes unbalanced, often indicated by acetate or ethanol formation (Mendoza-Vega *et al.* 1994). However, in the experiments performed, no ethanol or acetate production was observed. Nonetheless, the lower biomass produced at the higher glycerol feed rate as well as the lower yield suggest that the glycerol was diverted in a side reaction producing a hitherto unidentified by-product.

The biomass yield values obtained in the literature are quite similar. D'Anjou and Daugulis (2001), for instance reported a yield of 0.46 g/g using a $Mut^S$ strain grown on the fed-batch glycerol addition phase of their process. Hang *et al.* (2008) achieved a yield of 0.5 ± 0.04 g/g on glycerol fed-batch phase (both reports based on growth in an identically constituted medium to the one used in this study). Therefore, to determine the expected theoretical yield, a stoichiometric approach was used.

The stoichiometric formula for *Pichia* biomass was obtained by Carnicer *et al.* (2011) as $CH_{1.761}N_{0.143}O_{0.636}S_{0.0018}$which compares well to the averaged biomass formula of Roels, namely

$CH_{1.8}O_{0.5}N_{0.2}$. The composition for an untransformed $Mut^S$ strain was determined, with the slight changes in biomass composition due to transformation being assumed to be negligible. The oxygen concentration assumed for this stoichiometric ratio was 21% (i.e. when air is sparged).

$$C_3H_8O_3 + aO_2 + bNH_3 \longrightarrow cCH_{1.761}N_{0.143}O_{0.636}S_{0.0018} + dH_2O + eCO_2$$

No product formation was assumed on glycerol growth due to AOX repression by glycerol. The theoretical yield based on the above reaction equation was calculated to be 0.59 g/g where cell maintenance was assumed negligible. The absence of any observed intermediate carbon-containing compound (e.g. acetate, ethanol) suggested that the discrepancy between theoretical yield and actual yield may be due to some energy being used by the cell for maintenance or diverted in side reactions producing unidentified compounds.

The final volumes at the end of the fed-batch (glycerol) phase were approximately 4.1 L for the fed-batch processes operated at 0.17 /h and 3.9 L for fed-batch run 5 at a preset growth rate of 0.21 /h. The feed concentration of methanol was 200 g/L in a chemically defined medium. Methanol was fed until a maximum working volume of 5 L was reached. The average methanol feed rates are given in Table 4.9. The average biomass produced during the fed-batch runs was 63 ± 8 g/L. This represented an average yield of 0.45 ± 0.04 g/g. No protease activity was detected using the protease assay within the first 24 hours of methanol induction (results not shown). There was no residual methanol in the reactor during cultivation on methanol (detection limit ~0.1 g/L). The reported fed-batch strategies in the literature generally utilise a mixture of glycerol/methanol during the induction phase in order to boost biomass production during induction. D'Anjou *et al.* (2001), employed a glycerol batch phase before a glycerol/methanol fed-batch feed followed by a 100% methanol feed for 48 hours. This approach led to the production of 150-200 mg/L recombinant protein. In an alternative approach employed by Herwig *et al.* (2011), similar to the approach used above, methanol was added in pulses and the rate of depletion used to determine the specific rate of substrate uptake, $q_S$. The substrate was subsequently fed to the reactor at a rate equal to its uptake rate. This led to an increase in productivity of the recombinant protein synthesized compared to the fed-batch technique involving exponential feeding or constant methanol feeding in fed-batch fashion. Additionally, the authors obtained biomass yield values of between 0.3-0.4 g/g.

The approach used in these experiments allowed for the accurate, simple determination of the methanol utilisation rate and prevented its over-accumulation. The slow rate at which it was fed maintained high cell concentration and allowed a slight increase in the biomass. Due to the offline determination of product formation, it was difficult to examine the effect of the induction conditions

on the specific product formation rate. Thus it was not established whether the duration of induction, the concentration of methanol inducer or of residual methanol had a significant effect on the concentration of the final product.

Furthermore, it was not clear whether a residual methanol concentration was important for full activation of the AOX promoter. D'Anjou *et al.* (2001) ensured a 1-2 g/L residual methanol concentration in order to activate the promoter. Kupsculik *et al.*(2008)determined this concentration to be 0.45 g/L while Herwig *et al.* (2011) maintained undetectable levels of methanol. All authors recorded appreciable quantities of recombinant protein. In these experiments, methanol accumulation was prevented to minimise protease release. According to Sinha *et al.* (2000), despite the mechanism underlying protease release at high methanol concentrations being unknown, their investigations found a time dependent effect of methanol induction on protease release in a $Mut^+$ strain. It was not clear whether a similar effect existed for $Mut^S$ strains.

### 4.9 Approaches to detection of insulin precursor

#### 4.9.1 Planned approach to insulin recovery and identification

Identification and purification of the insulin precursor was attempted by various techniques described in Sections 3.9 and 3.10. An initial affinity purification step to purify the histidine-tagged insulin precursor from the proteins present in the extracellular milieu was used. The affinity purified fraction was to be identified and quantified on an HPLC Reverse Phase Column (Amersham, Pharmacia Biotech). Following this, processing of the precursor to produce purified insulin was to have involved trypsin-mediated cleavage of the precursor to remove the histidine tag. Further purification via HPLC would be required to remove the products of the enzymatic cleavage process. The insulin peak as detected would be fractionated off the HPLC column and quantified on a C-18 HPLC column.

#### 4.9.2 Efforts to identify insulin via HPLC

In order to identify the insulin precursor, insulin identification was attempted using the RPC column. The commercial insulin preparation (Lantus) was run on a SDS-PAGE gel to confirm the presence of insulin at the approximate size required (i.e 10-15 kDa) (Figure 4.23). The commercially obtained insulin sample was detectable on the gel down to a minimum concentration of 0.05 mg/ml. The extracellular sample, obtained from a bioreactor experiment in which cells had been induced by methanol for 48 hours, was subsequently run on a separate SDS-PAGE gel. A band corresponding to approximately 15 kDa, thought to be insulin precursor, was present on the gel. To further probe the presence of insulin, samples containing this fraction were run on the HPLC. In addition to insulin,

Lantus contained m-cresol that was likely to be identified by HPLC. In order to ascertain the peaks responsible for insulin, a pure solution of m-cresol was run separately on the column before running the commercial insulin preparation. From the peaks shown in Figure 4.24, Lantus had 3 distinct peaks corresponding to insulin, cresol and a peak due to use of the mobile phase.

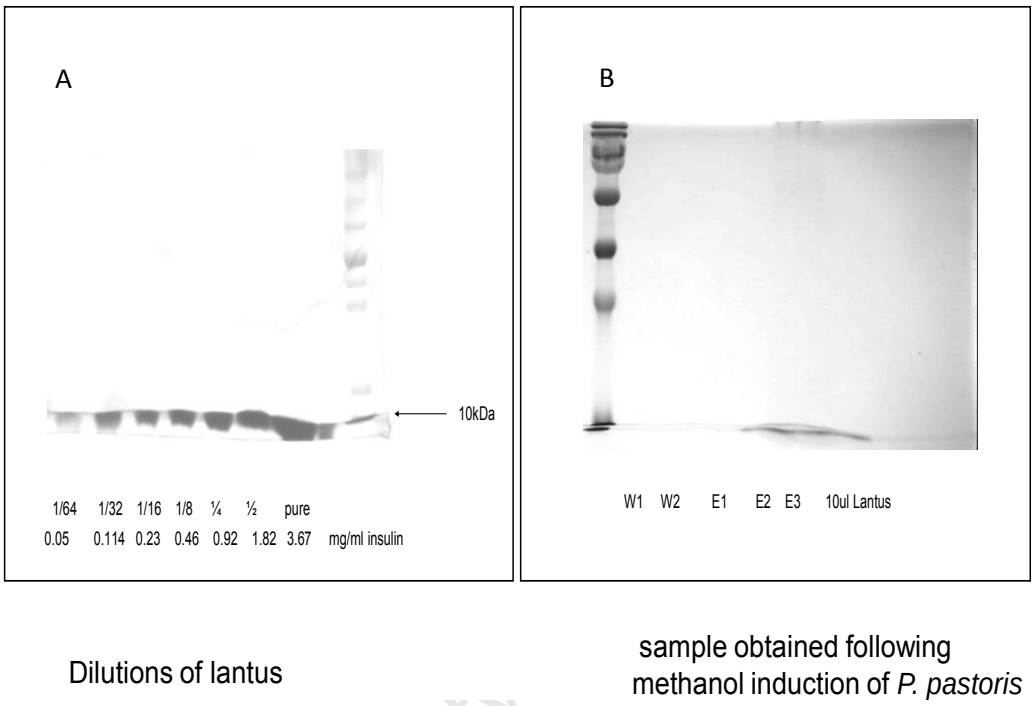


**Figure 4. 23 Dilutions of commercially available insulin molecule (A) run on a one-dimensional gel;(B) shows attempts to identify insulin precursor from samples obtained via affinity chromatography**
The commercially available insulin preparation Lantus (left hand side) was run at various dilutions on a 15 % SDS PAGE gel and visualised with Coomassie blue. The gel on the right hand side contains fractions obtained from an extracellular sample removed from a bioreactor experiment following induction by methanol for ~48 hours.

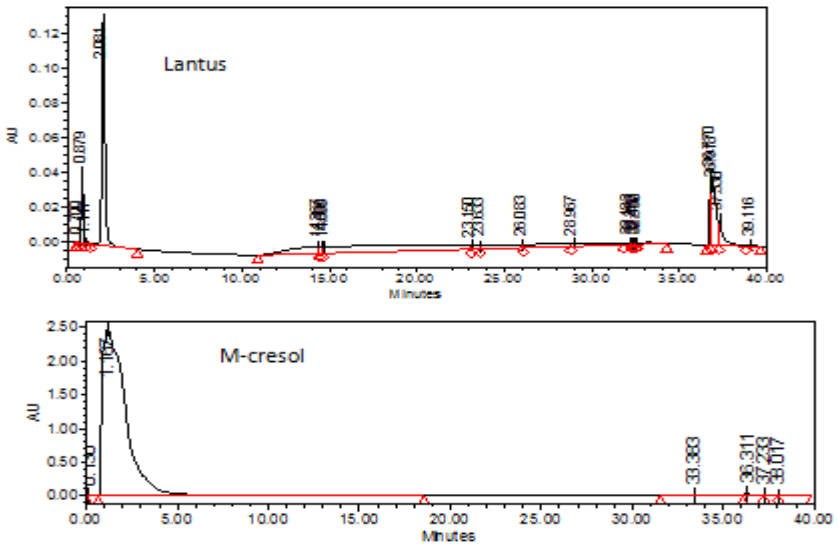


**Figure 4. 24 a Commercial insulin preparation containing a peak produced by m-cresol.**

Purified m-cresol subsequently run separately on the RPC column.

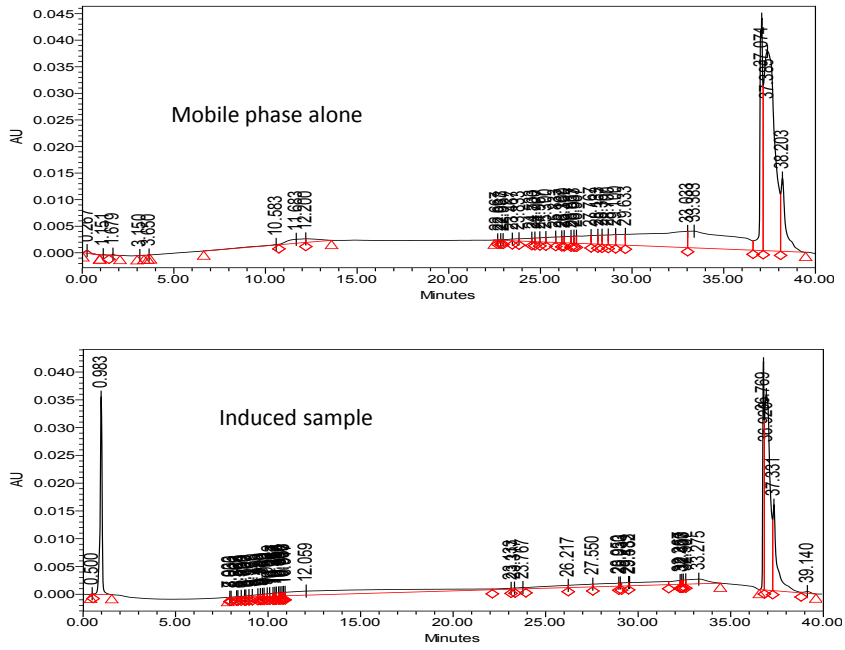


**Figure 4. 25HPLC profiles obtained when induced sample and mobile phase were run separately on RPC Column.**

Mobile phase appears to produce final peak in HPLC profile observed. From the results obtained in the induced sample profile, a peak potentially corresponding to insulin precursor appears at 0.983. Subsequent attempts to reproduce result were not promising.

Running m-cresol in mobile phase and comparing it to mobile phase only suggested that the peak at approximately 1.5 min was most likely due to m-cresol whereas the peak at approximately 30 minutes was due to mobile phase. Thus the peak produced just before m-cresol in the commercial insulin preparation, at approximately 1 minute, was proposed to be due to insulin. By this analysis, the peak produced in the induced sample at approximately 0.9 minutes may have been due to insulin precursor detection. However, attempts to reproduce this result were unsuccessful. Further, the absence of a pure insulin precursor standard as a positive control made it difficult to confirm this result.

### 4.9.4 Western blot to identify insulin precursor

The more precise Western blotting technique, based on antibody binding the precursor, was used to identify the insulin precursor. By means of a mouse-derived antibody raised against the insulin precursor, the presence of the protein was investigated. Additionally, as a control, the presence of a constitutively expressed protein (glyceraldehyde phosphate dehydrogenase-GAPDH) was also probed. The samples were not run through an affinity-purification process prior to antibody detection to avoid the possibility of the precursor being retained on the column. Additionally, cell lysates were obtained by ultra-sonication, to investigate the possibility that the insulin precursor was produced intracellularly but not secreted out of the cell. The lysate also contained GAPDH whose identification by Western blotting validated the use of the technique (Figure 4.26). However, a negative result, indicating no insulin precursor production was obtained when the membrane was stained with the anti-insulin precursor antibody (Figure 4.27).



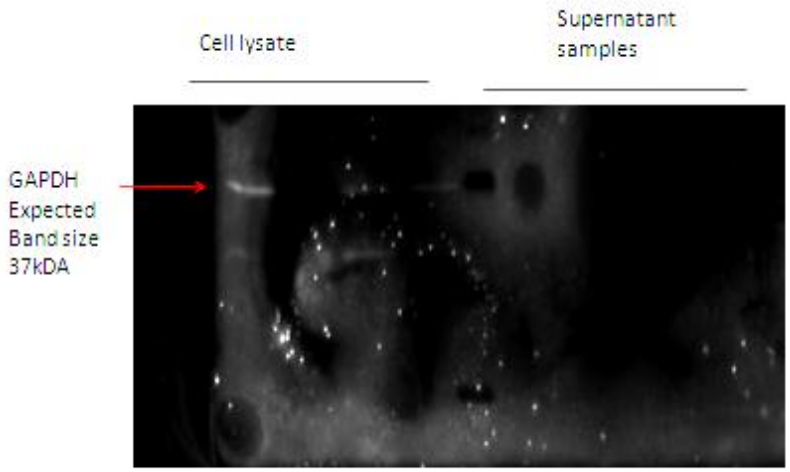


**Figure 4. 26Shows bands obtained when blot was stained with anti-GAPDH antibody. Bands indicated confirmed its presence.**

The first three lanes contain intracellular protein sample, the next three lanes contain samples from the extracellular matrix. As expected, the GAPDH protein is only visible in the intracellular protein samples.


**Figure 4.27 Negative result indicating non-detection of insulin precursor by Western Blot.**

The molecular weight marker (Fermentas Pre-stained PageRuler, SM 1811) indicates the region where the insulin precursor if present must lie. The absence of any band corresponding to this size indicates that insulin precursor is not present.

The absence of insulin precursor might have been due to the strain losing the plasmid for insulin expression. To investigate this, cells were removed at the start of cultivation, towards the end of the batch phase, in the fed-batch (glycerol) phase and during growth on methanol and cultured on agar plates containing the antibiotic geneticin. The plasmid encoding the insulin precursor contained a selection marker bearing geneticin resistance. In all cases, the cells grew successfully, indicating the presence of the plasmid. Thus it is unlikely that the lack of insulin precursor observed was due to loss of gene expression. An alternative reason for the lack of detection by Western Blotting may have been a result of the antibody not recognizing the insulin precursor due to differences in the sequence. Generally antibodies are highly specific, capable of recognizing between two enantiomers of a particular molecule. The specific antibody used in this experiment was a mouse antibody produced by Sigma Aldrich. The antibody was produced by injecting a synthetic human insulin precursor into mouse in order to elicit an immune response. The beta cell antibodies produced in the mouse with Immunoglobulin G anti-insulin precursor binding ability were harvested and used to detect insulin in this experiment. However it is possible that the insulin precursor used to raise antibodies in the mouse was slightly different in sequence to the insulin precursor produced in *P. pastoris*. Slight differences between the recombinant insulin precursor molecule, e.g. amino acid sequences, could prevent antibody-protein binding. To circumvent this uncertainty, further analysis used approaches that did not depend on antibody recognition.

### 4.9.5 MALDI-analysis

Matrix Assisted Laser Desorption/Ionization (MALDI) uses a laser bean to ionize trypsin-digested proteins embedded in a specific matrix. The ionized particles produce a spectrum within a pressurised vacuum known as the time-of-flight (TOF). The peak profiles produced by the proteins were subsequently entered into a database containing mass spectra for a large range of proteins. The match between the peak produced by the sample protein and peaks present in the databases was reported within a specific confidence interval, thus ascertaining with a high degree of certainty, the identity of the protein. Prior to the MALDI analysis, the protein harvested from the extracellular matrix of the bioreactor and from cell lysate was run on a SDS-PAGE (15%) gel. The bands produced were visualized. Based on molecular weight of the protein, putative bands potentially containing insulin were identified. The samples were subsequently prepared for MALDI analysis as described in Section 3.10. Together with the sample protein, two controls were used: the first was a positive control in the form of bovine serum albumin to verify the proper functioning of the instrument, the second contained trypsin in buffer solution (with no other protein added) to ensure that the peak profiles observed were not due to autolytic degradation of trypsin. The results obtained from the MALDI mass spectrometry facility are included in the Table 4.10.

**Table 4.10MALDI analysis results indicating proteins identified from *P. pastoris* fed-batch cultivation**

| Spot Index | Sample identification number | Protein identified | Protein score | % confidence interval |
|---|---|---|---|---|
| **B5** | BSA control | **Bovine Serum Albumin** | 871 | 100 |
| **B2** | Lane 1- (extracellular matrix) | **Ubiquitin** | 291 | 100 |
| **B3** | Lane 2(cell lysate) | **Neutral protease** | 163 | 100 |
| **B4** | Lane 3 (extracellular matrix) | **Polyubiquitin (Fragment)** | 234 | 74.6 |

Proteins identified with positive identifications in bold. Protein significance level is protein score = 75. Spot index refers to the sequence in which the sample proteins were placed on the matrix; Sample Identification number refers to the identity of the protein embedded in the matrix; the score indicates the match between the profile peak and the database values with a confidence interval given in the final column (based on information provided in report by Dr Mare Vlok, Center for Proteomics and Genomics Research, UCT Medical School).

A total of four samples were run in the MALDI Mass Spectrometer: a control containing Bovine Serum Albumin, two from the extracellular matrix (supernatant without cells) and one from the cell

lysate. As shown, the positive control was correctly identified as bovine serum albumin. In the experimental samples, ubiquitin and protease were detected with 100% confidence interval in the extracellular matrix and cell lysate respectively. Additionally a polyubiquitin complex was identified with a significantly high degree of confidence of 75%. It is further evident that insulin precursor was absent in all analysed samples. Protease expression is known to be a product of cell lysis. When toxic levels of methanol accumulate inside the cell and methanol is converted to hydrogen peroxide, this leads to rapid cell degradation resulting in the release of vacuolar proteases. Thus if cell lysis had occurred resulting in protease release hence insulin degradation, high quantities of protease would be expected in the extracellular matrix. However, the presence of protease within the cell lysate only indicates that the methanol feeding strategy used successfully circumvented vacuolar protease release due to methanol mediated cell lysis. The production of ubiquitin in the secreted protein in the extracellular matrix could not be attributed to the specific feeding strategy.

### 4.10 Discussion of MALDI data and possible reasons for absence of insulin precursor

### Putative Mechanism for Ubiquitin Mediated Insulin Degradation

Ubiquitin is a small protein present in all eukaryotic cells that is principally involved in directing the proper folding of cellular proteins. It binds to proteins that have not been properly folded and 'tags' them for destruction in the proteosome (Kimura & Tanaka 2010). It forms part of the complex protein folding machinery in eukaryotic cells (Gasser *et al.* 2008). Its role in *P. pastoris* has not been well elucidated. Based on observations made in *S. cerevisiae,* Sreekrishna *et al.* (1997) speculated that it plays a role in the proper folding of proteins and in secretion of proteins in *P. pastoris*(Sreekrishna *et al.* 1997). More recently, Vad and co-workers (2005) observed that a high amount of ubiquitin was co-secreted with a $Mut^S$ strain genetically engineered to secrete human parathyroid hormone. In general the high secretion of ubiquitin is typically associated with cellular stress (Gasser *et al.* 2008). A study of protein degradation in filamentous fungi by Kimura *et al.* (2011) found that heterologous protein is incorrectly identified as misfolded protein and targeted for degradation. While the same conclusion cannot be reached with certainty regarding the degradation of insulin precursor, a putative mechanism by which *P. pastoris* is "tagged"i.e. a molecular glycoprotein is attached to it which thus identifies it for degradation was speculated upon. The mechanism shown in Figure 4.24, adapts the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway given for proteolysis by ubiquitin in a typical *P. pastoris* cell. One method by which this pathway could be verified would be to conduct an experiment in which two separate experimental setups containing *P. pastoris* are cultivated and induced exactly, with one in the presence of protease inhibitors and the other without any protease inhibitors added. An investigation of the

recombinant protein levels in the two different conditions would reveal whether protease activity possibly involving the ubiquitin-mediated pathway was responsible for degradation of the recombinant protein.

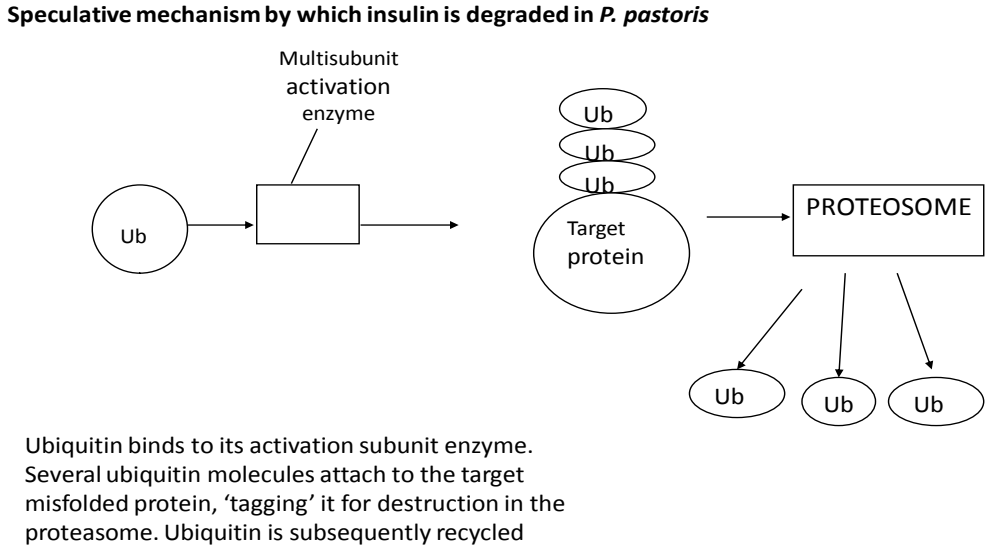


**Figure 4. 28 Putative mechanism for proteolytic degradation of insulin precursor in *P. pastoris***
(based on KEGG protocol, 2011).

## Error in translation of insulin precursor encoding gene as another factor for the absence of insulin precursor

The mechanism of insulin precursor production requires induction of the AOX 1 promoter resulting in the transcription of the insulin precursor gene to a complementary mRNA transcript. This mRNA transcript is targeted to the cytoplasmic ribosomal network for subsequent translation into protein for secretion. Thus an alternative hypothesis to the one provided in Section 4.9.6 might be that the absence of observed insulin precursor was due to lack of translation. This could be due to improper codon usage during the cloning of the gene, frame-shift mutations resulting in a transcript with 'nonsense' codons incorporated into the mRNA transcript. To validate this speculation, the total mRNA produced would have to be harvested and, using primers specific for insulin precursor encoding genes, probe the total mRNA for the presence of complementary base pairs.

## 4.11 Conclusions

Detailed conclusions of this thesis as they relate to the project's established aims are provided in Chapter 5. In summary however, the findings of this chapter indicate the following:

- Glycerol is an ideal carbon source for *P. pastoris* growth as it resulted in undetectable levels of ethanol production while supporting biomass growth.

- Negligibly low quantities of protease expression were detected in *P. pastoris* $Mut^S$ even at high residual methanol concentrations. The protease detected in the fed-batch experiments via MALDI analysis, being intracellular, is thus not the product of extensive cell lysis leading to vacuolar protease release as was initially predicted.

- The two-stage glycerol fed-batch process is an ideal, reproducible method of obtaining high biomass production in a large scale process. Its use of kinetic parameters obtained from batch data makes it scalable and applicable to a wide variety of systems. Further, at low methanol feed rates during the induction phase, biomass concentration can be maintained throughout the methanol fed-batch, due to slow growth of *P. pastoris* on methanol.

- Speculations regarding the absence of insulin precursor suggest either lack of translation or the degradation of insulin precursor via the ubiquitin mediated proteosome.

# Chapter 5

# Conclusions and Recommendations

*P. pastoris* has become a widely used vector for recombinant protein production mainly due to its strongly and tightly regulated methanol inducible alcohol oxidase 1 (AOX1) promoter. Insulin production in *P. pastoris* is the subject of much investigation and its commercial use for insulin production has been reported by Biocon India (Biocon 2011b). Attempts to design a fed-batch process for insulin production in *P. pastoris* have focussed on iterative approaches and improvements in downstream purification. Such studies have limited scalability as they require empirical strategies for process design. The aim of this project was therefore to design a fed-batch experimental process using batch-derived parameters. This would allow a high biomass concentration to be obtained before induction of gene expression by methanol addition in fed-batch mode. The optimised fed-batch conditions (media, carbon source, dissolved oxygen concentration) were to be determined in batch experiments. Methanol induction conditions were determined in batch shake flask experiments and tested in a preliminary fed-batch run. Three optimised fed-batch experiments were conducted and the biomass and substrate utilisation profiles observed. Efforts to identify insulin precursor in the extracellular and intracellular regions were unsuccessful due to possible degradation of insulin precursor by the cellular proteasome or errors in mRNA translation. The broad conclusions derived from this study as well as recommendations for further investigation are given below.



### 5.1 Conclusions

Following from the aims of the study presented in Section 2.14, the following conclusions were drawn from this study:

- In general, biomass production on glycerol and glucose in chemically defined medium was higher than that achieved in complex medium. The average shake flask values for chemically defined medium and complex medium were 3.7 and 4.9 g/L respectively. The average biomass concentrations on glucose in the bioreactor were 21 g/L and 29 g/L respectively
- While cells grew at higher specific growth rates, producing higher biomass concentrations in shake flasks containing glucose, the production of ethanol reduced the suitability of glucose

as a carbon source. This was due to the repressive nature of ethanol on the AOX promoter reported in the literature. No ethanol production was observed during growth on glycerol, thus making it the carbon source of choice for subsequent bioreactor experiments.

- The bioreactor experiment investigating growth on glycerol in complex medium revealed a slow rate of glycerol utilisation during the early phase of growth. During this period it was likely that cells utilised the yeast-extract and peptone for growth. A chemically defined medium was therefore optimised for fed-batch operation. The key kinetic parameters derived from the batch bioreactor studies were the $\mu_{max}$, $Y_{X/S}$ and average maximum biomass values. These were 0.22 ± 0.02 /h, 0.5 ± 0.01 g/g and 29 ± 2.5 g/L respectively.
- Shake flask experiments to investigate the effect of methanol concentration on protease activity showed that *P. pastoris* produced a low activity of extracellular protease during batch cultivation in shake flask medium. Subsequent experiments to determine growth and methanol utilisation in shake flask experiments did not yield useful data. It was likely that the growth observed was due to yeast extract and peptone present in the inoculum carried over into the experimental medium. However, the experiments conducted showed that a methanol concentration of 20 g/L and above would result in growth inhibition of *P. pastoris*. For this reason, the fed-batch bioreactor experiments aimed to maintain a residual methanol concentration of no more than 10 g/L methanol.
- Fed-batch experiments based on the batch derived kinetic parameters showed highly reproducible batch conditions. The approach was useful in generating significant increases in biomass during the fed-batch growth on glycerol. In three optimised runs conducted at preset growth rates of 0.17, 0.17 and 0.21 /h, the increases in biomass during the fed-batch phases were 148, 152 and 110 g respectively and the preset growth rates were achieved.
- Based on an empirically derived rate of methanol utilisation from both shake flask and bioreactor experiments, an optimal methanol feed rate of approximately 7 g/Lh was determined. In the three optimised fed-batch runs, no methanol accumulation was subsequently observed. Methanol feed rates of 3.8, 2.8 and 2.94 g/Lh resulted in slight biomass production at specific growth rates of 0.006, 0.002 and 0.005 /h.
- Downstream processing experiments to purify and identify the insulin precursor in order to further process it to produce insulin were unsuccessful. The Western blotting experiment designed to identify the insulin precursor using an antibody did not detect the recombinant protein.
- MALDI analysis revealed that a protease and ubiquitinase were released during the advanced stages (over 50 hours) into the methanol induction phase. This suggests that the

insulin precursor may have been degraded by the proteases released into the extracellular matrix.

- Additionally, errors in translation may have resulted in the absence of insulin precursor.

## 5.2 Recommendations

### 5.2.1 Use of glucose as a carbon source

Biomass production was greatly enhanced by using glucose as a carbon substrate. It was not used in subsequent scale-up experiments however, due to the production of ethanol upon growth on glucose. Ethanol was thought to repress the AOX promoter, hence potentially diminishing the recombinant potential of *P. pastoris.* However, recent reports of recombinant protein production in *P. pastoris* grown on glucose in chemically defined medium suggest that with some modifications, glucose can be used to cultivate *P. pastoris* on glucose(Heyland *et al.* 2010). Heyland *et al.* (2010) utilised glucose to study metabolic pathways within *P. pastoris*. While the authors observed ethanol production its effect was minimal due to a very high dissolved oxygen concentration which prevented anaerobic processes resulting in ethanol production from taking place. Additionally, they used and the utilisation of a GAP promoter instead of an AOX promoter minimsed the potential repressive effects of ethanol. Thus in the event of ethanol production, repression would be unlikely as the GAP promoter was not reported to be repressed by ethanol.

The recent development of a new cultivation system for glucose-limited fed-batch cultivation without the need for external feed of substrate may have significant implications for *P. pastoris* cultivation. The method developed by Panula-Peroda *et al.* (2010) relies on the gradual release of glucose into the medium following enzymatic degradation of a starch supplied via continuous diffusion from a storage gel in proportion to the biomass increase. The new storage gel is known as EnBase and EnBase-controlled cultures have repeatedly achieved cell densities 5 to 20 times that achieved in standard cultivations. Krause *et al.* (2001) report increases in cell concentrations without any concomitant reduction in productivity of standard cultures. However, no reports of EnBase use in *P. pastoris* cultivation have been reported to date.

### 5.2.2 Use of a protease-deficient strain

In order to prevent the possibility of proteolytic degradation of the insulin precursor, a protease-deficient strain could be used. Often this is an optimal strategy if other methods of reducing proteolysis in high cell concentration cultures prove futile. Protease deficient strains are reported to be difficult to transform and do not grow vigorously, however(Cereghino *et al.* 2002).

### 5.2.3 Use of a protease inhibitor

In order to investigate whether protease release alone is responsible for the degradation of insulin precursor, it is proposed that two fed-batch experiments be conducted. In one of the experiments, a quantity of protease inhibitor must be added during the methanol induction phase, while the protease inhibitor is not added in the control. From this it could be established whether protease release alone was responsible for the lack of detectable insulin in the reactor. While the use of a commercial protease inhibitor is not viable for industrial processes, it would provide a useful indication of whether a protease-deficient strain would be more ideally suited for the production of insulin precursor in *P. pastoris*.

### 5.2.4 Investigation into time and concentration dependent protease release

The exact mechanism by which protease release from*P.pastoris* occurs is not known. As mentioned earlier, in addition to being a response to high residual methanol concentrations, it also appears to be due to long periods of exposure to residual methanol concentrations. In the experiments conducted, no residual methanol was present. The protease release observed in the MALDI analysis, therefore, might be a result of time-dependent protease release. In order to investigate this, regular assaying of protease ought to be conducted during the methanol induction phase. By determining the specific time point beyond which protease release occurs in the presence of methanol, it might be possible to design a methanol induction phase that does not expose cells to methanol for longer than necessary.

### 5.2.5 Use of sorbitol as a co-substrate during methanol induction phase

The use of sorbitol as a co-substrate during the induction of gene expression by methanol was initially proposed for the *P. pastoris* $Mut^S$ strain Brierley *et al.*( 1990). It is a non-repressing carbon source. Therefore biomass concentration can be increased without sorbitol inhibiting methanol induction. Consequently, this may enhance product formation. The use of sorbitol as a co-substrate in cultivation of a $Mut^+$ strain resulted in a 1.4 fold increase in biomass yield, 1.2 fold reduction in protease activity and a 50 mg/L increase in recombinant protein production in one report published by Celik *et al.* (2009). Thorpe *et al.* (1999) showed that a $Mut^S$ strain grown on sorbitol/methanol mixtures achieved lower growth rates than those achieved on glycerol/methanol co-substrate fed-batch but achieved higher specific rates of product formation. However, while it is known that *P. pastoris* simultaneously consumes sorbitol and methanol (Jungo *et al.* 2007), the biomass yields upon sorbitol and methanol in a fed-batch process would not necessarily be the sum of the yields of *P. pastoris* on the separate carbon sources. Thus it is important to determine the biomass yields and

productivity in the presence of both sorbitol and methanol. To do this it was suggested that a chemostat setup be used (D'Anjou, personal communication). Using a Design-of-Experiments approach, the cell concentration could be varied by altering the dilution rate and varying the inlet methanol/sorbitol feed. The dilution rate could be varied between 0.01 to 0.1 /h and the ratio of methanol: sorbitol varied from 10:1 to 1:2 ensuring that for each combination, the amount of carbon added is the same. This would generate a response surface for cell yield and productivity. From this, the best methanol/sorbitol ratio could be determined. Using the values for biomass yield and productivity, an exponential feed on sorbitol/methanol can be conducted using the same approach employed in the exponential glycerol fed-batch phase.

In conclusion therefore, this study sets out an approach with potential application in large scale processes. However, prior to this, further investigations outlined here ought to be conducted.

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# Appendices

## Appendix A: Confirmation of the presence of gene encoding insulin precursor

The ability of the mutant strain provided by the International Center for Genetic Engineering and Biotechnology (ICGEB) to produce the insulin precursor was probed to investigate whether the gene encoding the human insulin precursor was present and in-frame. To do this, DNA was harvested, cloned into a strain of *E. coli*containing very little DNA of its own (the DH5 α strain) for DNA amplification. The DNA subsequently harvested was sent to a sequencing facility. The results obtained confirmed the presence of the insulin precursor gene. The sequence obtained also showed that the DNA sequence was in-frame and did not contain any mutations.Subsequent DNA sequencing confirmed the presence of the gene (with no mutations).

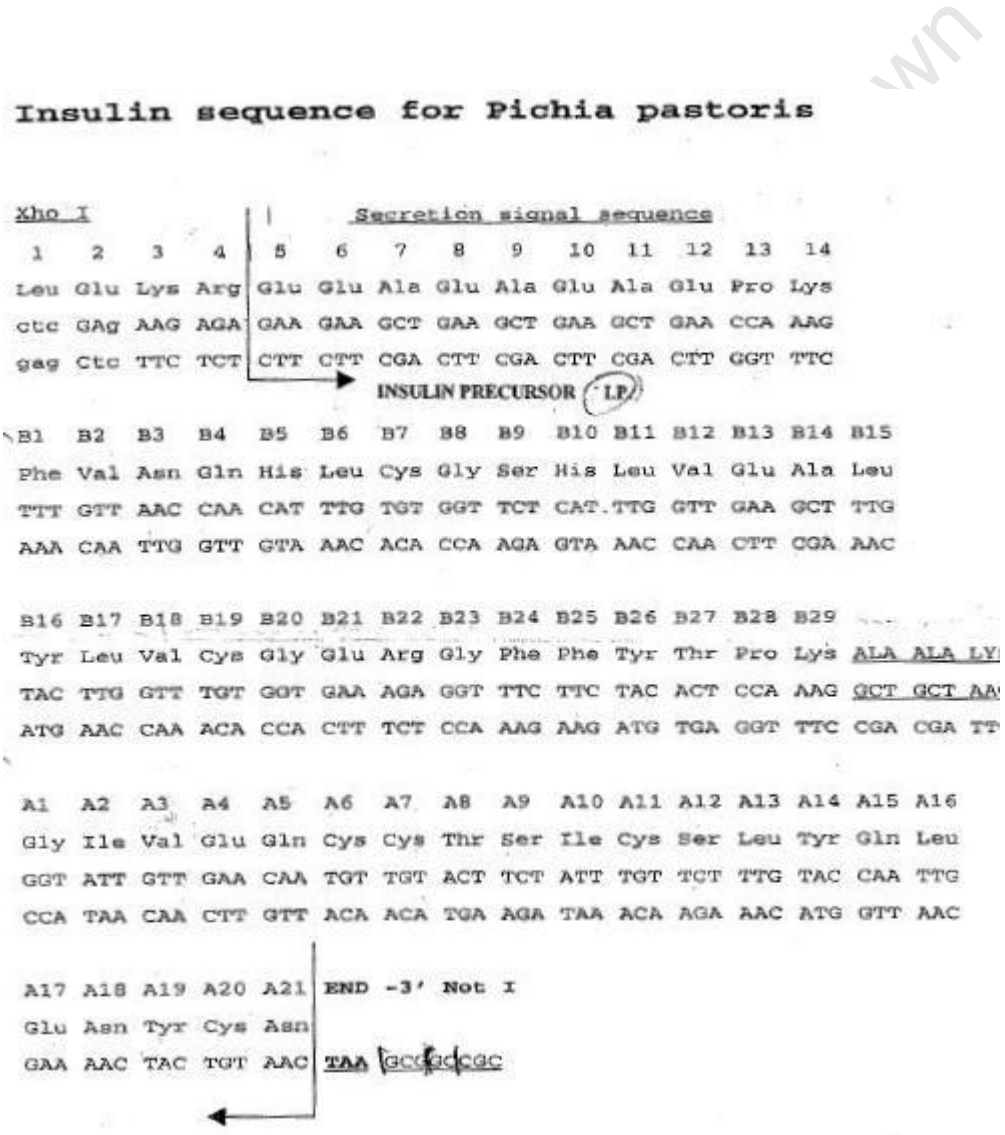


**Figure A. 1 DNA base sequence for gene encoding human insulin precursor provided by the International Center for Genetic Engineering and Biotechnology.**

## Appendix B Correlations used to determine cell dry weight based on optical density

In order to obtain a value for the cell dry weight, a correlation between measured cell concentration at a wavelength of 600 nm ($OD_{600}$) and the gravimetric cell dry weight was obtained. From this correlation subsequent $OD_{600}$ values were converted into measurements of Cell Dry Weights. The correlation below was obtained as follows:

i. *P. pastoris* cells were prepared in yeast-extract peptone medium (pre-inoculum) for 24 hours.
ii. A 10 % v/v inoculum was used to inoculate a shake flask medium containing chemically defined medium containing glycerol (50 g/L).
iii. Samples were withdrawn periodically from the flask and cell dry weight and $OD_{600}$ values were measured.

a. *Cell dry weight*

- To measure cell dry weight, a 2 ml volume of medium was centrifuged in a pre-dried, pre-weighed Eppendorf tube at maximum speed for 5 minutes. The supernatant was discarded
- The pellet obtained was dried at $80^0$C for 24 hours
- The Eppendorf tube containing the pellet was dried in a vacuum container for an additional 2-4 hours prior to weighing
- The mass of the Eppendorf tube containing the cell pellet was measured on a 4-decimal place balance. The Eppendorf tube was dried under vacuum for a further 4-5 hours and the mass checked until a constant reading of weight was obtained, indicating a value for cell dry weight in the absence of moisture.

b. *$OD_{600}$*

- The 2 ml cell suspension withdrawn was mixed by gently tapping the side of the Eppendorf tube.
- Before taking a reading, the spectrophotometer was set to the desired wavelength (600 nm) and 'blanked' to ensure that any change in absorbance readings was due to the presence of cells not due to absorbance of the medium components. The spectrophotometer was blanked using either distilled water (when cells were grown in chemically defined medium) or using 1% yeast-extract, 2% peptone solution (when cells were grown on YP medium).
- After blanking, a 1 ml volume of cell suspension was added to a glass cuvette and a reading was taken.

- Absorbance varies with cell concentration in linear fashion according to Beer-Lambert's law. Beyond a critical value of cell concentration, the linear relationship between cell concentration and OD does not hold. To determine this point, a highly dense cell suspension was diluted using distilled water to obtain a range of differently diluted cell suspensions (from 1:5 to 1:1). The values obtained allowed are shown below.

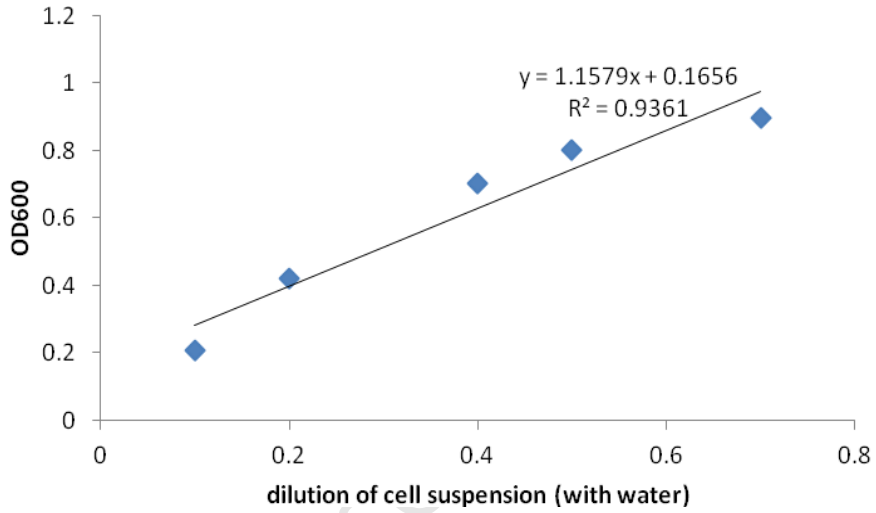


**Figure A. 2 Correlation between linear absorbance and cell concentration.**
The dilutions were correlated with observed cell concentration measurements. Linearity appears to breakdown after OD values of 0.7-0.8. The measurements were taken in triplicate.

From the graph it was seen that linearity broke down after OD values were above 0.7. Thus in subsequent experiments, OD values were maintained below 0.7. The OD/biomass correlations used for all the shake flask **cell concentration** cell concentration

The correlation shown in Figure A.4 was used in calculating the biomass from OD values.

In general the calculation was

$$Biomass\ \left(\frac{g}{L}\right) = OD_{600x}0.664$$

## Appendix C HPLC analysis

The protocol for HPLC analysis was described in Section 3.9.3. For each of the analytes detected for, a number of standard curves were prepared. The average curve is presented. Additionally, from a series of standard curves of glycerol, a general analysis of variance of the HPLC instrument is presented. Additional information is supplied where necessary.

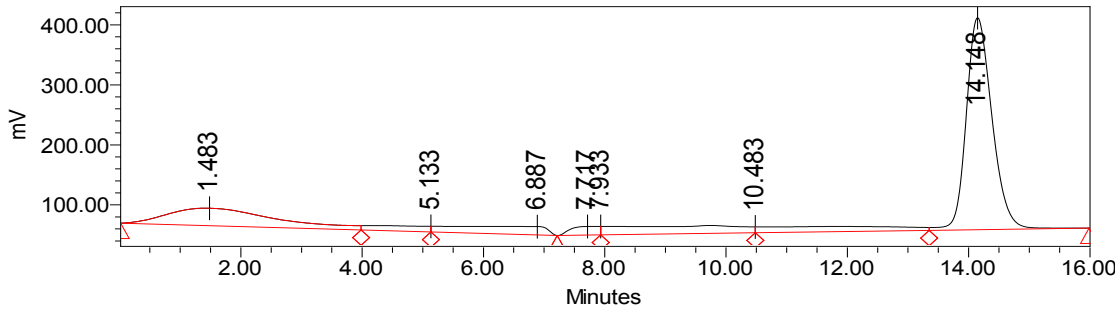


**Figure A. 3Typical glycerol chromatogram**

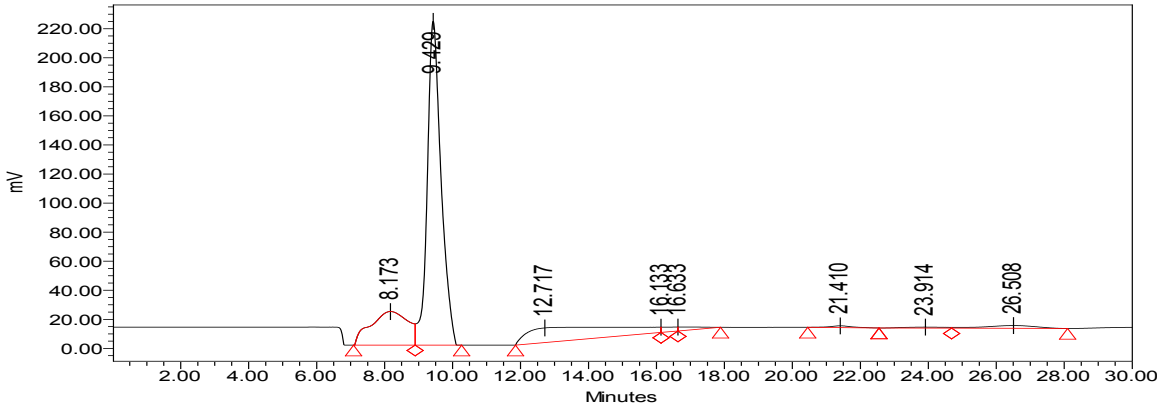


**Figure A. 4 Typical glucose chromatogram (8 g/L)**

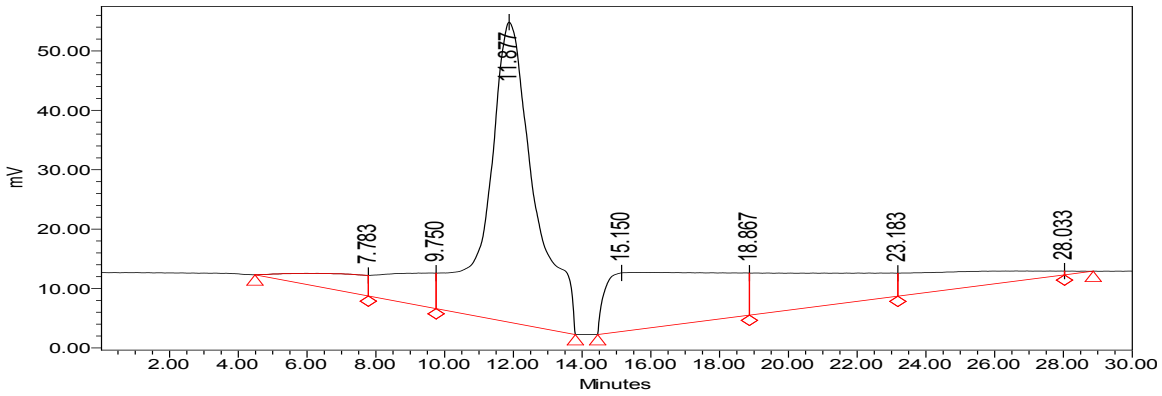


**Figure A. 5Typical ethanol chromatogram**

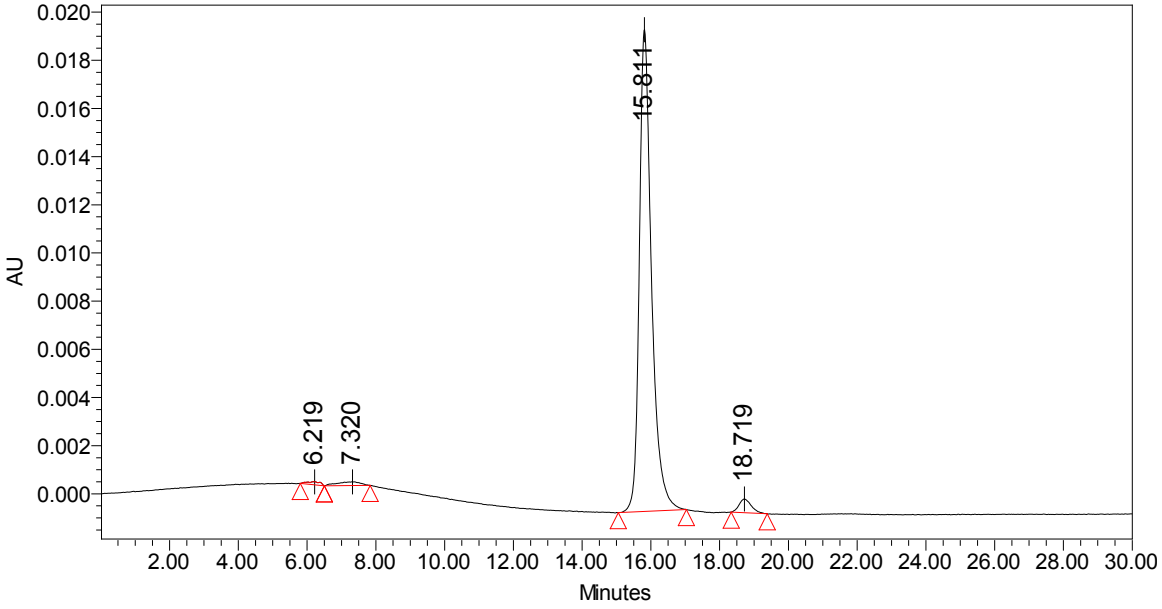


**Figure A. 6 Typical acetic acid chromatogram**

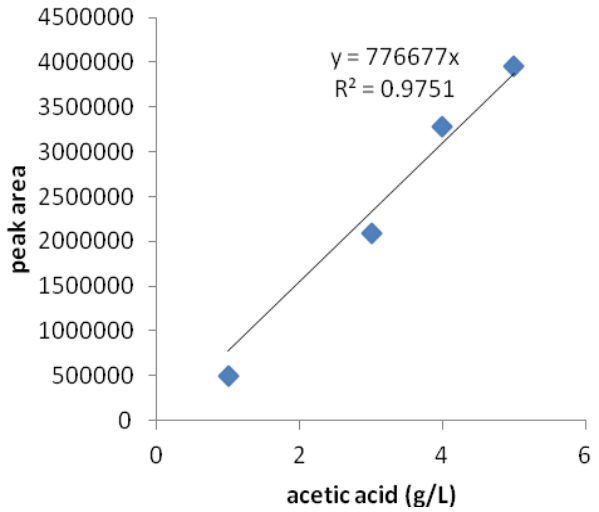


**Figure A. 7 Acetic acid standard curve**

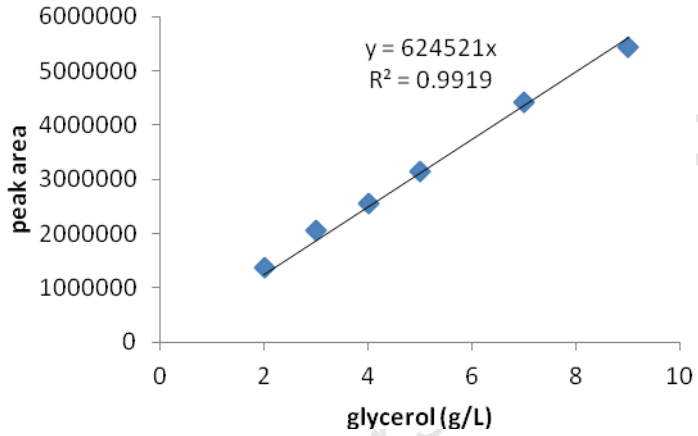


**Figure A. 8 Glycerol standard curve**

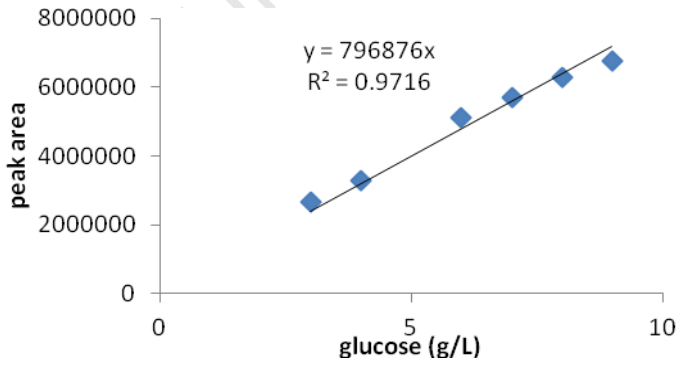


**Figure A. 9 glucose standard curve**

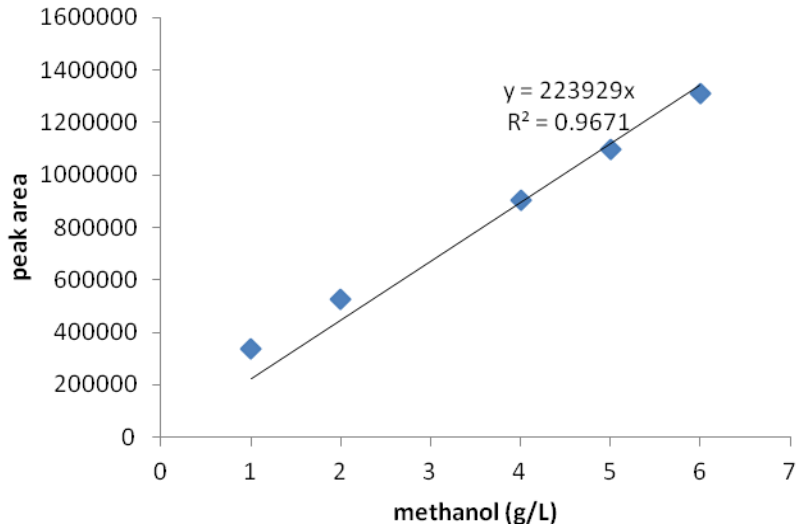


**Figure A. 10 methanol standard curve**

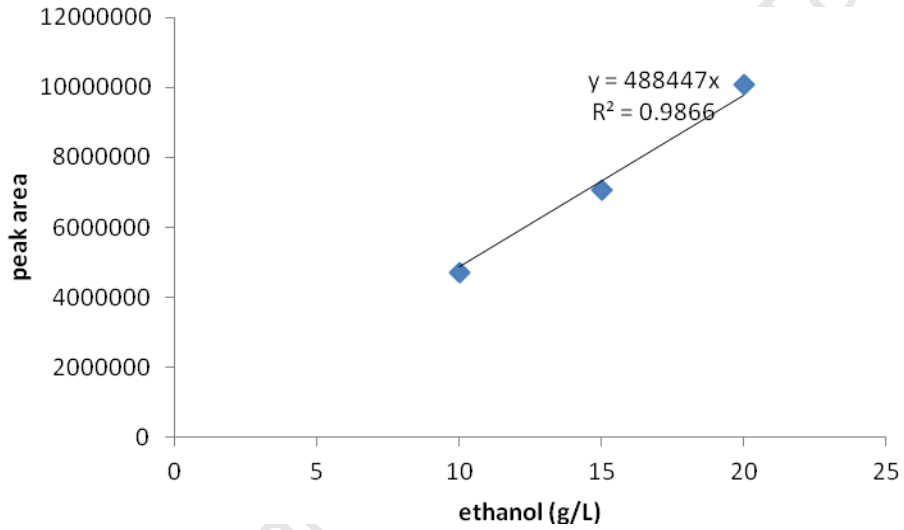


**Figure A. 11 Ethanol standard curve**

**Table B. 1 Standard retention times for compounds identified**

| Compound | Peak retention time (min) |
|---|---|
| **Glucose** | 9.425 |
| **Glycerol** | 14.163 |
| **Ethanol** | 11.877 |
| **Acetate** | 15.77(Absorbance at 274 nm) |
| **Methanol** | 19.400 |

## C 2 Determining Error in HPLC measurement

To determine error in the HPLC analysis, set concentrations of glycerol (10 g/L) were measured in triplicate. The standard deviation from the average peak area was used to calculate the percentage error intrinsic to the instrument's measurement.

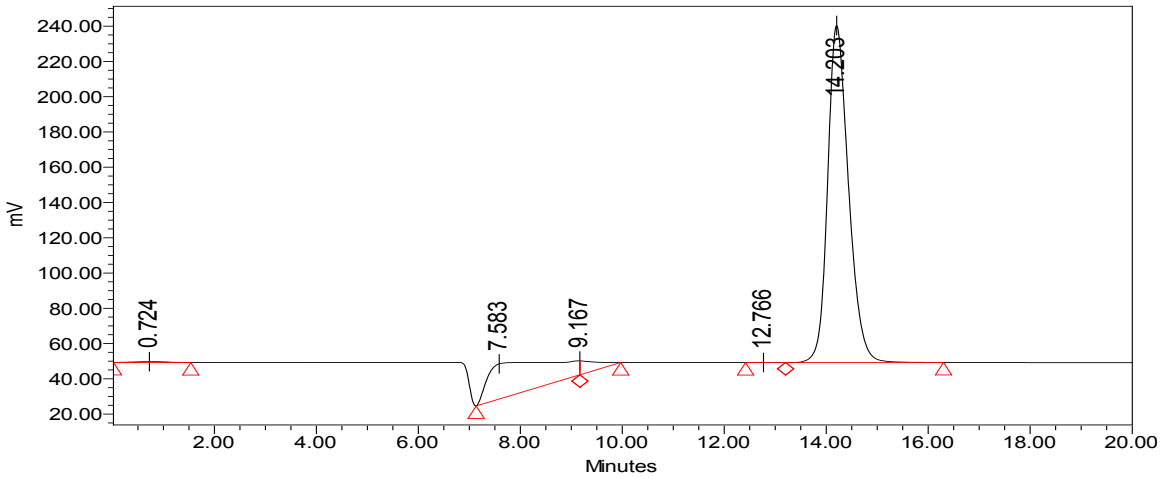


**Figure A. 12 HPLC peak produced when 10 g/L glycerol is run on a Biorad column**

**Table C. 1 Determination of error in HPLC measurement. Three separate glycerol runs with peak area values given**

| Glycerol concentration (g/L) | Peak area 1 | Peak area 2 | Peak area 3 | Average peak area | Standard Deviation | % Standard Deviation |
|---|---|---|---|---|---|---|
| **10** | 5442979 | 5858536 | 5574877 | 5625464 | 212346.9 | 3.77 |

From the measurement shown the percentage error in the measurement of glycerol concentration is approximately 4 %.

## Appendix D Growth data from shake flask and bioreactor experiments

This appendix presents the growth and substrate utilisation data obtained when *P. pastoris* was cultured in chemically defined and complex medium with glycerol, glucose and methanol as carbon sources. The experiments on shake flask experiments were performed in triplicate (n=3). The bioreactor data obtained in complex medium was from a single experiment.

### D1 Growth on glycerol in chemically defined medium containing glycerol (50 g/L) (Fig 4.1)

**Table D1. 1growth in chemically defined medium containing 50 g/L glycerol**

| Time | Cdw1 | Cdw2 | Cdw3 | Average | Stdev |
|---|---|---|---|---|---|
| **0** | 0.89 | 0.81 | 0.93 | 0.88 | 0.06 |
| **2** | 1.23 | 1.15 | 1.27 | 1.22 | 0.06 |
| **4** | 1.32 | 1.28 | 1.35 | 1.32 | 0.03 |
| **6** | 1.61 | 1.57 | 1.60 | 1.60 | 0.02 |
| **8** | 1.88 | 1.93 | 1.97 | 1.93 | 0.04 |
| **10** | 2.44 | 2.34 | 2.45 | 2.41 | 0.06 |
| **12** | 2.65 | 2.71 | 2.90 | 2.75 | 0.13 |
| **24** | 5.22 | 5.82 | 5.28 | 5.44 | 0.33 |
| **36** | 5.48 | 5.48 | 5.44 | 5.47 | 0.02 |

**Table D1. 2 Table with OD values for growth on glycerol over 36 hours in chemically defined medium**

| Time (hrs) | OD1 | OD2 | OD3 | Average (OD) | Standard Deviation |
|---|---|---|---|---|---|
| **0** | 0.46 | 0.41 | 0.46 | 0.45 | 0.03 |
| **2** | 0.63 | 0.58 | 0.63 | 0.62 | 0.03 |
| **4** | 0.68 | 0.65 | 0.67 | 0.67 | 0.02 |
| **6** | 0.83 | 0.80 | 0.79 | 0.81 | 0.02 |
| **8** | 0.97 | 0.98 | 0.97 | 0.97 | 0.00 |
| **10** | 1.26 | 1.18 | 1.21 | 1.22 | 0.04 |
| **12** | 1.36 | 1.37 | 1.44 | 1.39 | 0.04 |
| **24** | 2.69 | 2.94 | 2.62 | 2.75 | 0.17 |
| **36** | 2.82 | 2.77 | 2.70 | 2.76 | 0.06 |

**Table D1. 3 glycerol concentrations according to HPLC measurements**

| time | [glycerol 1] (g/L) | [glycerol 2] (g/L) | [glycerol 3] (g/L) | average | stdev |
|---|---|---|---|---|---|
| **0** | 59 | 52 | 55 | 55 | 4 |
| **2** | 46 | 50 | 44 | 47 | 3 |
| **4** | 48 | 48 | 37 | 44 | 6 |
| **6** | 38 | 37 | 31 | 35 | 4 |
| **8** | 26 | 26 | 24 | 26 | 1 |
| **10** | 20 | 20 | 24 | 22 | 2 |
| **12** | 20 | 13 | 12 | 15 | 4 |
| **24** | 16 | 8 | 8 | 10 | 5 |
| **36** | 5 | 5 | 6 | 5 | 0 |

## D2 Growth in chemically defined medium containing glucose (50 g/L) (Fig 4.2)

**Table D2. 1 growth in chemically defined medium containing glucose (50 g/L)**

| time | OD1 | OD2 | OD3 | average | standard dev |
|---|---|---|---|---|---|
| **0** | 0.296 | 0.292 | 0.345 | 0.311 | 0.030 |
| **2.5** | 0.709 | 0.761 | 0.899 | 0.790 | 0.098 |
| **5.5** | 1.435 | 1.110 | 1.260 | 1.268 | 0.163 |
| **7** | 1.395 | 1.170 | 1.645 | 1.403 | 0.238 |
| **9.5** | 1.930 | 1.590 | 2.500 | 2.007 | 0.460 |
| **11** | 1.785 | 2.710 | 3.505 | 2.667 | 0.861 |
| **24** | 3.395 | 4.070 | 4.425 | 3.963 | 0.523 |
| **26** | 3.420 | 4.280 | 4.720 | 4.140 | 0.661 |
| **30** | 3.725 | 4.685 | 4.830 | 4.413 | 0.601 |
| **48** | 4.233 | 4.679 | 4.935 | 4.616 | 0.355 |

**Table D2. 2 CDW data for growth on glucose (50g/L) in chemically defined medium**

| Time (hours) | CDW 1 | CDW 2 | CDW 3 | average | standard dev |
|---|---|---|---|---|---|
| **0** | 0.91 | 0.68 | 0.67 | 0.75 | 0.05 |
| **2.5** | 1.67 | 1.27 | 1.73 | 1.56 | 0.23 |
| **5.5** | 3.01 | 2.00 | 2.43 | 2.48 | 0.27 |
| **7** | 3.35 | 2.51 | 3.17 | 3.01 | 0.35 |
| **9.5** | 4.21 | 2.86 | 4.82 | 3.96 | 0.99 |
| **11** | 5.76 | 5.31 | 6.76 | 5.95 | 0.73 |
| **24** | 8.40 | 8.25 | 8.54 | 8.39 | 0.15 |
| **26** | 8.67 | 8.13 | 9.11 | 8.63 | 0.49 |
| **30** | 8.89 | 8.18 | 9.32 | 8.80 | 0.57 |
| **48** | 10.10 | 8.17 | 9.20 | 9.16 | 0.58 |

**Table D2. 3 glucose concentrations over time**

| Time (hours) | [glucose 1] (g/L) | [glucose 2] (g/L) | [glucose 3] (g/L) | Average (g/L) | standard dev |
|---|---|---|---|---|---|
| **0** | 43 | 41 | 40 | 41 | 2 |
| **2.5** | 39 | 40 | 35 | 38 | 3 |
| **5.5** | 38 | 40 | 32 | 36 | 4 |
| **7** | 37 | 40 | 31 | 36 | 4 |
| **9.5** | 37 | 39 | 31 | 36 | 4 |
| **11** | 36 | 38 | 31 | 35 | 4 |
| **24** | 28 | 26 | 24 | 26 | 2 |
| **26** | 25 | 24 | 21 | 24 | 1 |
| **30** | 15 | 14 | 13 | 14 | 1 |
| **48** | 3 | 3 | 2 | 3 | 1 |

**Table D2. 4 Ethanol production during growth in chemically defined medium containing glucose (50 g/L)**

| Time (hours) | [ethanol] (g/L) | [ethanol] (g/L) | [ethanol] (g/L) | average | stdev |
|---|---|---|---|---|---|
| **0** | 1.1 | 0.9 | 1.8 | 1.3 | 0.5 |
| **2.5** | 8.6 | 9.2 | 5.4 | 7.8 | 2.0 |
| **5.5** | 6.7 | 6.8 | 4.5 | 6.0 | 1.3 |
| **7** | 4.0 | 4.2 | 2.7 | 3.6 | 0.9 |
| **9.5** | 3.4 | 3.3 | 2.3 | 3.0 | 0.7 |
| **11** | 2.3 | 2.4 | 0.1 | 1.6 | 1.3 |
| **24** | 0.2 | 1.8 | 0.0 | 0.7 | 1.0 |
| **26** | 0.3 | 0.0 | 0.0 | 0.1 | 0.2 |
| **30** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **48** | 1.1 | 0.9 | 1.8 | 1.3 | 0.5 |

## D 3 Growth in chemically defined medium containing methanol (50 g/L) (Fig 4.3)

**Table D3. 1growth on methanol (49 g/L) in chemically defined medium**

| time | OD1 | OD2 | OD3 | average | standard dev | standard dev |
|---|---|---|---|---|---|---|
| 0 | 0.34 | 0.33 | 0.38 | 0.35 | 0.03 | 0.07 |
| 2.5 | 0.64 | 0.62 | 0.67 | 0.64 | 0.03 | 0.10 |
| 5.5 | 0.66 | 0.66 | 0.66 | 0.66 | 0.00 | 0.08 |
| 7 | 0.73 | 0.70 | 0.86 | 0.76 | 0.09 | 0.18 |
| 9.5 | 0.76 | 0.79 | 0.78 | 0.78 | 0.02 | 0.06 |
| 11 | 0.84 | 0.86 | 0.93 | 0.88 | 0.04 | 0.09 |
| 24 | 0.91 | 1.22 | 1.15 | 1.08 | 0.16 | 0.21 |
| 26 | 1.16 | 1.20 | 1.21 | 1.19 | 0.03 | 0.09 |
| 30 | 1.20 | 1.32 | 1.10 | 1.21 | 0.11 | 0.23 |
| 36 | 1.21 | 1.34 | 1.18 | 1.24 | 0.09 | 0.24 |

**Table D3. 2 methanol utilisation for cells grown in chemically defined medium containing 49 g/L methanol**

| time | [methanol 1](g/L) | [methanol 2] (g/L) | [methanol3](g/L) | average (g/L) |
|---|---|---|---|---|
| **0** | 43 | 46 | 46 | 45 |
| **2.5** | 43 | 42 | 40 | 42 |
| **5.5** | 43 | 41 | 39 | 41 |
| **7** | 38 | 39 | 40 | 39 |
| **9.5** | 36 | 38 | 39 | 38 |
| **11** | 35 | 36 | 38 | 36 |
| **24** | 32 | 36 | 35 | 34 |
| **26** | 30 | 35 | 35 | 33 |
| **30** | 28 | 31 | 32 | 30 |
| **36** | 26 | 29 | 29 | 28 |

**Table D3. 3 Cell dry weight (g/L) for growth in chemically defined medium containing methanol (50g/L)**

| Time (hours) | CDW 1 | CDW 2 | CDW 3 | average | Standard dev |
|---|---|---|---|---|---|
| **0** | 0.75 | 0.65 | 0.77 | 0.72 | 1.57 |
| **2.5** | 1.41 | 1.22 | 1.36 | 1.33 | 1.37 |
| **5.5** | 1.46 | 1.31 | 1.33 | 1.37 | 1.71 |
| **7** | 1.60 | 1.37 | 1.73 | 1.57 | 1.20 |
| **9.5** | 1.67 | 1.56 | 1.58 | 1.60 | 1.35 |
| **11** | 1.85 | 1.70 | 1.87 | 1.81 | 1.29 |
| **24** | 2.00 | 2.40 | 2.31 | 2.24 | 2.04 |
| **26** | 2.55 | 2.36 | 2.44 | 2.45 | 2.53 |
| **30** | 2.64 | 2.61 | 2.22 | 2.49 | 1.85 |
| **36** | 2.64 | 2.62 | 2.22 | 2.49 | 1.43 |

## D 4 Growth in complex medium

### *D 4.1 Growth in complex medium containing glycerol (40 g/L) (Figure 4.6)*

**Table D4. 1 growth in complex medium containing glycerol (40g/L)**

| time | OD1 | OD 2 | OD 3 | average | stdev |
|---|---|---|---|---|---|
| **0** | 0.52 | 0.46 | 0.50 | 0.49 | 0.03 |
| **4** | 0.78 | 0.84 | 0.79 | 0.80 | 0.03 |
| **8** | 2.32 | 2.00 | 1.80 | 2.04 | 0.26 |
| **12** | 3.14 | 2.90 | 2.90 | 2.98 | 0.14 |
| **24** | 6.10 | 5.86 | 5.88 | 5.95 | 0.13 |
| **32** | 7.37 | 7.69 | 7.47 | 7.51 | 0.16 |
| **48** | 8.91 | 8.96 | 8.07 | 8.65 | 0.50 |

**Table D4. 2 growth on glycerol in complex medium (40g/L)**

| time | CDW 1 | CDW 2 | CDW 3 | average | stdev | stdev(%) |
|---|---|---|---|---|---|---|
| **0** | 0.31 | 0.28 | 0.30 | 0.30 | 0.02 | 5.92 |
| **4** | 0.47 | 0.51 | 0.48 | 0.48 | 0.02 | 4.35 |
| **8** | 1.39 | 1.20 | 1.08 | 1.23 | 0.16 | 12.77 |
| **12** | 1.89 | 1.75 | 1.74 | 1.79 | 0.08 | 4.61 |
| **24** | 3.67 | 3.53 | 3.54 | 3.58 | 0.08 | 2.24 |
| **32** | 4.43 | 4.63 | 4.50 | 4.52 | 0.10 | 2.17 |
| **48** | 5.36 | 5.39 | 4.86 | 5.21 | 0.30 | 5.78 |

**Table D4. 3 glycerol utilisation in complex medium**

| Time (h) | glycerol (g/L) | glycerol (g/L) | glycerol (g/L) | Average (g/L) | Stdev (g/L) |
|---|---|---|---|---|---|
| **0** | 40 | 44 | 41 | 42 | 2 |
| **4** | 40 | 40 | 40 | 40 | 0 |
| **8** | 37 | 39 | 36 | 37 | 1 |
| **12** | 37 | 35 | 33 | 35 | 2 |
| **24** | 10 | 12 | 10 | 11 | 1 |
| **48** | 0 | 0 | 0 | 0 | 0 |

## *D 4.2 Growth in complex medium containing glucose (40 g/L)*

**Table D4. 4 Growth of *P. pastoris*in complex medium containing 40 g/L glucose (figure 4.7)**

| time | OD 1 | OD 2 | OD 3 | average | CDW 1 (g/L) | CDW 2 (g/L) | CDW 3 (g/L) | Average (g/L) | Stdev (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| **0** | 0.94 | 0.65 | 0.59 | 0.73 | 0.56 | 0.39 | 0.36 | 0.44 | 0.11 |
| **2** | 1.33 | 0.96 | 0.79 | 1.02 | 0.80 | 0.58 | 0.47 | 0.62 | 0.17 |
| **4** | 2.34 | 1.84 | 1.54 | 1.90 | 1.41 | 1.10 | 0.93 | 1.15 | 0.24 |
| **5.5** | 2.84 | 2.98 | 2.39 | 2.74 | 1.71 | 1.79 | 1.44 | 1.65 | 0.19 |
| **8** | 4.01 | 4.04 | 3.13 | 3.72 | 2.41 | 2.43 | 1.88 | 2.24 | 0.31 |
| **10** | 4.77 | 4.89 | 4.33 | 4.66 | 2.87 | 2.94 | 2.61 | 2.81 | 0.18 |
| **12** | 5.94 | 5.90 | 5.13 | 5.65 | 3.57 | 3.55 | 3.09 | 3.40 | 0.27 |
| **24** | 6.97 | 7.15 | 7.17 | 7.10 | 4.19 | 4.30 | 4.32 | 4.27 | 0.07 |
| **36** | 7.11 | 7.28 | 7.38 | 7.26 | 4.28 | 4.38 | 4.44 | 4.37 | 0.08 |

**Table D4. 5Glucose utilisation in complex medium**

| time | glucose 1 | glucose 2 | glucose 3 | average glu | stdevglu | average eth | stdev(eth) |
|---|---|---|---|---|---|---|---|
| 0 | 42 | 40 | 40 | 41 | 1 | 0 | 0 |
| 4 | 40 | 38 | 37 | 38 | 1 | 0 | 0 |
| 8 | 38 | 32 | 34 | 35 | 3 | 3 | 2 |
| 12 | 36 | 31 | 29 | 32 | 3 | 6 | 1 |
| 24 | 20 | 26 | 28 | 25 | 4 | 13 | 6 |
| 36 | 10 | 10 | 12 | 11 | 1 | 10 | 5 |

**Table D4. 6Ethanol production during growth in complex medium containing 40 g/L glucose**

| Time (hours) | ethanol 1 | ethanol 2 | ethanol 3 |
|---|---|---|---|
| **0** | 0 | 0 | 0 |
| **4** | 0 | 0 | 0 |
| **8** | 5 | 4 | 1 |
| **12** | 5 | 6 | 7 |
| **24** | 11 | 20 | 8 |
| **36** | 12 | 14 | 5 |

### *D 4.3 Growth in complex medium containing methanol (40 g/L) for Figure 4.8*

**Table D4. 7 Growth on yp methanol (40g/L)**

| time | Average OD1 | ln(OD1) | Average OD2 | ln(OD2) | Average OD3 | ln(OD3) | average |
|---|---|---|---|---|---|---|---|
| **0** | 1.21 | 0.20 | 0.82 | -0.19 | 1.25 | 0.22 | 0.66 |
| **2** | 1.25 | 0.22 | 1.34 | 0.29 | 1.28 | 0.24 | 0.87 |
| **4** | 1.93 | 0.66 | 1.95 | 0.67 | 1.89 | 0.63 | 1.42 |
| **5.5** | 2.10 | 0.74 | 2.26 | 0.82 | 2.38 | 0.86 | 1.66 |
| **8** | 2.62 | 0.96 | 2.73 | 1.00 | 2.77 | 1.02 | 2.02 |
| **10** | 3.33 | 1.20 | 3.19 | 1.16 | 3.21 | 1.16 | 2.42 |
| **12** | 3.43 | 1.23 | 3.50 | 1.25 | 3.59 | 1.28 | 2.60 |
| **24** | 4.38 | 1.48 | 4.64 | 1.53 | 4.76 | 1.56 | 3.36 |
| **36** | 4.57 | 1.52 | 4.47 | 1.50 | 4.65 | 1.54 | 3.34 |

**Table D4. 8 cell dry weight on yp methanol (40 g/L)**

| time | CDW 1 | CDW 2 | CDW 3 | average | stdev |
|---|---|---|---|---|---|
| **0** | 0.50 | 0.75 | 0.73 | 0.66 | 0.14 |
| **2** | 0.80 | 0.77 | 0.75 | 0.77 | 0.03 |
| **4** | 1.17 | 1.13 | 1.16 | 1.16 | 0.02 |
| **5.5** | 1.36 | 1.43 | 1.27 | 1.35 | 0.08 |
| **8** | 1.64 | 1.66 | 1.58 | 1.63 | 0.05 |
| **10** | 1.92 | 1.93 | 2.00 | 1.95 | 0.05 |
| **12** | 2.10 | 2.16 | 2.06 | 2.11 | 0.05 |
| **24** | 2.79 | 2.87 | 2.63 | 2.76 | 0.12 |
| **36** | 2.69 | 2.80 | 2.75 | 2.75 | 0.05 |

| time | methanol 1 | methanol 2 | methanol 3 | average | stdev |
|---|---|---|---|---|---|
| **0** | 45 | 41 | 43 | 43 | 2 |
| **4** | 43 | 42 | 42 | 42 | 1 |
| **8** | 35 | 40 | 39 | 38 | 2 |
| **12** | 36 | 39 | 40 | 38 | 2 |
| **24** | 34 | 40 | 39 | 38 | 3 |
| **36** | 34 | 39 | 35 | 36 | 3 |

## Appendix E Bioreactor data

**Table E. 1 Growth of *P. pastoris* in complex medium containing 40 g/L glycerol**

| time | OD1 | OD2 | dilution factor | OD average | ln OD | peak area | gly conc | CDW g/L | ln (CDW) |
|---|---|---|---|---|---|---|---|---|---|
| **0** | 0.268 | 0.269 | 1 | 0.27 | -1.315 | 1771498 | 42.2 | 0.162 | -1.822 |
| **1** | 0.472 | 0.458 | 1 | 0.47 | -0.766 | 1921459 | 42.1 | 0.28 | -1.273 |
| **2** | 0.535 | 0.541 | 1 | 0.54 | -0.62 | 2026405 | 41.4 | 0.324 | -1.127 |
| **3** | 0.559 | 0.564 | 1 | 0.56 | -0.577 | 2057703 | 41.4 | 0.338 | -1.084 |
| **4** | 0.677 | 0.67 | 1 | 0.67 | -0.395 | 2056572 | 40.8 | 0.406 | -0.902 |
| **5** | 0.365 | 0.359 | 2 | 0.72 | -0.323 | 2097050 | 40.6 | 0.436 | -0.83 |
| **6** | 0.468 | 0.454 | 2 | 0.92 | -0.081 | 2092757 | 40.6 | 0.555 | -0.588 |
| **7** | 0.112 | 0.116 | 10 | 1.14 | 0.131 | 2006707 | 40.4 | 0.687 | -0.376 |
| **8** | 0.728 | 0.738 | 2 | 1.47 | 0.383 | 1838142 | 37.0 | 0.883 | -0.125 |
| **9** | 0.588 | 0.569 | 4 | 2.31 | 0.839 | 1672335 | 33.6 | 1.393 | 0.331 |
| **10** | 0.666 | 0.685 | 4 | 2.70 | 0.994 | 1544194 | 31.1 | 1.627 | 0.487 |
| **11** | 0.76 | 0.781 | 5 | 3.85 | 1.349 | 1483569 | 29.8 | 2.32 | 0.842 |
| **12** | 0.536 | 0.535 | 10 | 5.36 | 1.678 | 1327478 | 26.7 | 3.225 | 1.171 |
| **13** | 0.661 | 0.674 | 10 | 6.68 | 1.898 | 1157957 | 23.3 | 4.02 | 1.391 |
| **14** | 0.423 | 0.453 | 20 | 8.76 | 2.17 | 1093541 | 22.0 | 5.275 | 1.663 |
| **15** | 0.483 | 0.48 | 20 | 9.63 | 2.265 | 940083 | 18.9 | 5.799 | 1.758 |
| **16** | 0.546 | 0.532 | 20 | 10.78 | 2.378 | 896221 | 18.0 | 6.492 | 1.871 |
| **24** | 0.379 | 0.374 | 50 | 18.83 | 2.935 | 273137 | 5.5 | 11.337 | 2.428 |
| **26** | 0.46 | 0.496 | 50 | 23.90 | 3.174 | 232500 | 4.7 | 14.394 | 2.667 |
| **30** | 0.587 | 0.567 | 50 | 28.85 | 3.362 | 98214 | 2.0 | 17.375 | 2.855 |
| **32** | 0.68 | 0.672 | 50 | 33.80 | 3.52 | 66675 | 1.3 | 20.356 | 3.013 |
| **36** | 0.68 | 0.72 | 50 | 35.00 | 3.555 | 35611 | 0.7 | 21.078 | 3.048 |
| **48** | 0.299 | 0.301 | 100 | 30.00 | 3.401 | 0 | 0.0 | 20.031 | 2.997 |

**Appendix E2 Bioreactor experiment to investigate growth in chemically defined medium containing glycerol for Figure 4.11**

The bioreactor experiments were conducted as described in chapter 3. The first OD and CDW values were obtained as described earlier in Appendix B. These values were subsequently used to calculated CDW 2 and 3 from the OD2 and OD 3 values shown below. Dissolved oxygen fluctuated between each time interval. However the values obtained at hourly points were largely representative of the values obtained between samples.

**Table E2. 1 Growth of *P. pastoris*in chemically defined medium containing 50 g/L glycerol**

| time | OD1 | OD2 | OD3 | average | stdev | CDW1 | CDW2 | CDW3 | average |
|---|---|---|---|---|---|---|---|---|---|
| **0** | 0.90 | 0.84 | 1.05 | 0.93 | 0.11 | 0.57 | 0.45 | 0.69 | 0.57 |
| **2** | 1.34 | 1.03 | 1.14 | 1.17 | 0.16 | 0.79 | 0.59 | 0.76 | 0.71 |
| **4** | 1.40 | 1.21 | 1.40 | 1.34 | 0.11 | 0.83 | 0.69 | 0.92 | 0.81 |
| **6** | 1.94 | 1.53 | 1.88 | 1.78 | 0.22 | 1.14 | 0.88 | 1.24 | 1.09 |
| **8** | 2.70 | 3.40 | 1.96 | 2.69 | 0.72 | 1.59 | 1.95 | 1.30 | 1.61 |
| **12** | 3.53 | 4.44 | 2.96 | 3.65 | 0.75 | 2.08 | 2.54 | 1.96 | 2.19 |
| **14** | 4.81 | 5.03 | 4.16 | 4.67 | 0.45 | 2.83 | 2.88 | 2.76 | 2.82 |
| **16** | 9.71 | 9.78 | 9.58 | 9.69 | 0.10 | 5.71 | 5.60 | 6.35 | 5.89 |
| **18** | 13.79 | 14.01 | 13.57 | 13.79 | 0.22 | 8.11 | 8.03 | 8.99 | 8.38 |
| **20** | 17.21 | 15.98 | 16.47 | 16.55 | 0.62 | 10.12 | 9.16 | 10.91 | 10.06 |
| **22** | 27.98 | 28.87 | 26.35 | 27.73 | 1.28 | 16.45 | 16.54 | 17.46 | 16.82 |
| **24** | 40.40 | 42.30 | 41.75 | 41.48 | 0.98 | 23.75 | 24.24 | 27.67 | 25.22 |
| **26** | 48.40 | 47.32 | 48.58 | 48.10 | 0.68 | 28.45 | 27.12 | 32.19 | 29.25 |
| **36** | 49.20 | 48.70 | 49.50 | 49.13 | 0.40 | 28.92 | 27.91 | 32.80 | 29.88 |

**Table E2. 2 Glycerol and dissolved oxygen concentration during *P. pastoris* growth in chemically defined medium containing 50 g/L glycerol**

| glycerol 1 | glycerol 2 | glycerol 3 | average | stdev | DO1 | DO2 | DO3 | average | stdev | time |
|---|---|---|---|---|---|---|---|---|---|---|
| **51.223** | 49.9 | 52.2 | 51.1 | 1.1 | 91.2 | 100.0 | 95.0 | 95.4 | 4.4 | 0 |
| **45.755** | 47.8 | 48.4 | 47.3 | 1.4 | 90.0 | 93.3 | 91.0 | 91.4 | 1.7 | 2 |
| **44.901** | 43.7 | 46.6 | 45.1 | 1.4 | 89.5 | 85.9 | 87.0 | 87.5 | 1.8 | 4 |
| **44.716** | 43.0 | 44.3 | 44.0 | 0.9 | 85.4 | 78.9 | 84.0 | 82.8 | 3.4 | 6 |
| **39.630** | 40.7 | 43.9 | 41.4 | 2.2 | 80.0 | 74.0 | 82.0 | 78.7 | 4.2 | 8 |
| **39.364** | 35.8 | 40.8 | 38.6 | 2.6 | 68.5 | 57.0 | 75.0 | 66.8 | 9.1 | 12 |
| **38.453** | 30.3 | 34.9 | 34.5 | 4.1 | 54.3 | 49.3 | 68.4 | 57.3 | 9.9 | 14 |
| **35.997** | 24.3 | 32.9 | 31.1 | 6.0 | 52.2 | 44.6 | 50.8 | 49.2 | 4.0 | 16 |
| **28.198** | 14.2 | 23.0 | 21.8 | 7.1 | 38.2 | 37.2 | 43.5 | 39.6 | 3.4 | 18 |
| **20.762** | 12.9 | 13.9 | 15.9 | 4.3 | 19.0 | 17.0 | 12.0 | 16.0 | 3.6 | 20 |
| **15.442** | 6.0 | 3.9 | 8.4 | 6.1 | 2.5 | 0.6 | 1.9 | 1.7 | 1.0 | 22 |
| **10.157** | 3.1 | 2.7 | 5.3 | 4.2 | 48.4 | 57.0 | 65.0 | 56.8 | 8.3 | 24 |
| **0.289** | 0.3 | 0.0 | 0.2 | 0.2 | 63.0 | 72.0 | 75.9 | 70.3 | 6.6 | 26 |
| **0.197** | 0.0 | 0.0 | 0.1 | 0.1 | 74.0 | 79.0 | 75.2 | 76.1 | 2.6 | 36 |

## Appendix F Methanol shake flask data

Three major shake flask experiments were performed to determine the growth of *P. pastoris* on methanol: growth on YP-glycerol followed by methanol addition; growth in chemically defined medium containing glycerol followed by the addition of methanol and finally growth in chemically defined medium containing methanol alone.

The raw data for each of the experiments is presented below.

### F1 Calibrating the Protease Assay

The protease assay was calibrated as described in Section 3.9.4. The calibration curve is presented in Figure A.14.

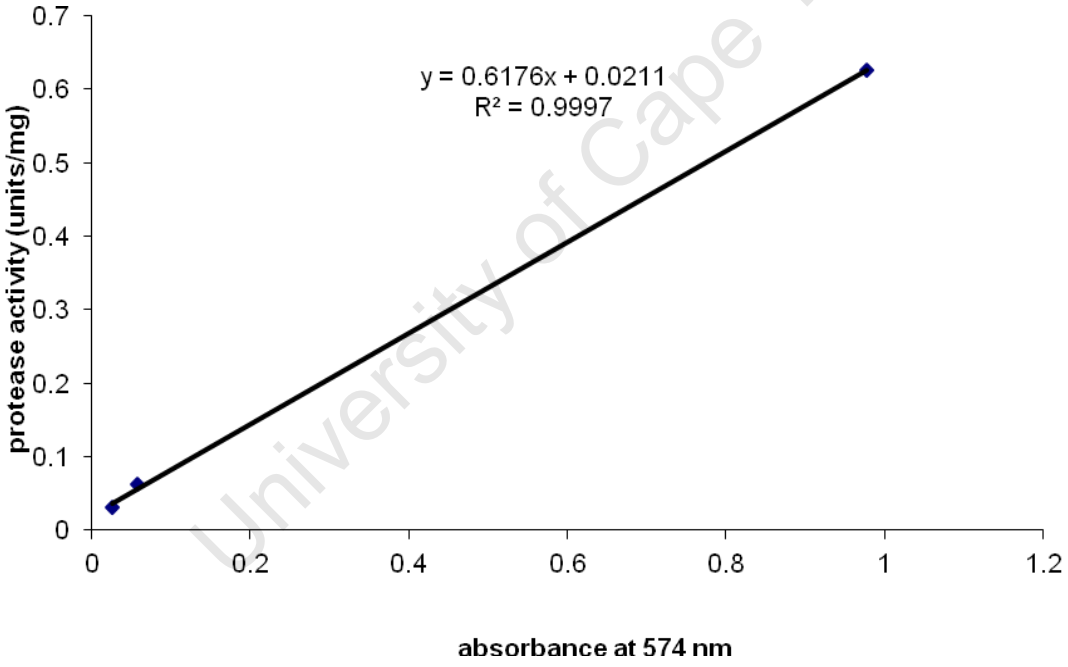




**Figure A. 13 calibration curve: absorbance due to proteinase K**

**Table F. 1growth data on cells grown on YP glycerol before methanol addition at various concentrations for Figure 4.14**

| **Yp Alone** | | | **YPM(10 g/L)** | **OD** | **ln(OD)** |
|---|---|---|---|---|---|
| **time** | OD | ln(OD) | time | | |
| 0 | 9.13 | 2.21 | 0 | 10.3 | 2.33 |
| 2 | 11.2 | 2.42 | 2 | 10.26 | 2.33 |
| 4 | 11.66 | 2.46 | 4 | 9.91 | 2.29 |
| 7 | 11.28 | 2.42 | 7 | 9.48 | 2.25 |
| 9 | 12.45 | 2.52 | 22 | 12.73 | 2.54 |
| 22 | 13.38 | 2.59 | 48 | 14 | 2.64 |
| 48 | 12.875 | 2.56 | | | |
| **YPM(1 g/L)** | | | **YPM(20 g/L)** | | |
| | | | | OD | ln(OD) |
| **time** | OD | ln(OD) | time | | |
| 0 | 11.3 | 2.42 | 0 | 10.49 | 2.35 |
| 2 | 12.07 | 2.49 | 2 | 10.17 | 2.32 |
| 4 | 12.15 | 2.50 | 4 | 10.24 | 2.33 |
| 7 | 11.9 | 2.48 | 9 | 11.4 | 2.43 |
| 9 | 12.17 | 2.50 | 22 | 12.52 | 2.53 |
| 22 | 12.59 | 2.53 | 48 | 13.125 | 2.57 |
| 48 | 12.25 | 2.51 | | | |
| **YPM(4 g/L)** | | | **YPM(40 g/L)** | | |
| | | | | OD | ln(OD) |
| **time** | OD | ln(OD) | time | | |
| 0 | 11.3 | 2.42 | 0 | 9.99 | 2.30 |
| 2 | 11.4 | 2.43 | 2 | 9.69 | 2.27 |
| 4 | 11.35 | 2.43 | 4 | 9.27 | 2.23 |
| 7 | 12.62 | 2.54 | 7 | 8.51 | 2.14 |
| 9 | 12.3 | 2.51 | 9 | 9.22 | 2.22 |
| 22 | 13.4 | 2.60 | 22 | 12.1 | 2.49 |
| 48 | 10.775 | 2.38 | 48 | 12.2 | 2.50 |

**Table F. 2protease activity determined by measuring absorbance and accounting for absorbance due to Yeast-Peptone (Figure 4.12)**

| Absorbance at 574 nm | absorbance due to yeast-peptone | actual absorbance | Protease activity (units/mg) |
|---|---|---|---|
| **0.017** | 0.003 | 0.014 | 0.0297464 |
| **0.022** | 0.003 | 0.019 | 0.0346872 |
| **0.052** | 0.003 | 0.049 | 0.0532152 |
| **0.058** | 0.003 | 0.055 | 0.0569208 |
| **0.078** | 0.003 | 0.075 | 0.0692728 |
| **0.088** | 0.003 | 0.085 | 0.0754488 |

## Appendix G Growth on glycerol prior to methanol addition for Figure 4.13

Subsequently cells were grown on glycerol for 24 hours followed by methanol addition for a further 24 hours. The raw data for that experiment are presented below. The glycerol concentrations listed indicate the initial glycerol concentration values prior to methanol addition.

### G 1 Residual glycerol concentrations at the end of 24 hours growth

**Table G1. 1 cell growth on glycerol prior to methanol addition**

| time | OD1 | OD2 | dil factor | Average | corrected | ln (OD) | CDW (g/L) |
|---|---|---|---|---|---|---|---|
| 0 | 0.305 | 0.296 | 1 | 0.3005 | 0 | -1.20 | 0 |
| 8 | 0.814 | 0.809 | 1 | 0.8115 | 0.51 | -0.208 | 0.992 |
| 24 | 0.251 | 0.251 | 10 | 2.51 | 2.20 | 0.920 | 4.29 |
| 26 | 0.274 | 0.279 | 10 | 2.765 | 2.46 | 1.01 | 4.78 |
| 30 | 0.36 | 0.349 | 10 | 3.545 | 3.24 | 1.26 | 6.30 |
| 34 | 0.37 | 0.37 | 10 | 3.7 | 3.39 | 1.30 | 6.60 |
| 36 | 0.41 | 0.413 | 10 | 4.115 | 3.81 | 1.41 | 7.40 |
| 48 | 0.505 | 0.506 | 10 | 5.055 | 4.75 | 1.62 | 9.23 |
| 54 | 0.512 | 0.533 | 10 | 5.225 | 4.92 | 1.65 | 9.56 |

**Table G1. 2 Growth after methanol addition at 24 hours**

| time | OD1 | OD2 | dil factor | Average | normalized | Ln (normalized od) | Normalized (CDW) Based on correlation |
|---|---|---|---|---|---|---|---|
| **0** | 0.45 | 0.45 | 1.00 | 0.45 | 0.00 | -0.80 | 0.00 |
| **8** | 0.76 | 0.75 | 1.00 | 0.76 | 0.31 | -0.28 | 0.59 |
| **24** | 0.25 | 0.28 | 10.00 | 2.65 | 2.20 | 0.97 | 4.26 |
| **26** | 0.29 | 0.28 | 10.00 | 2.86 | 2.41 | 1.05 | 4.67 |
| **30** | 0.32 | 0.33 | 10.00 | 3.28 | 2.83 | 1.19 | 5.50 |
| **34** | 0.36 | 0.37 | 10.00 | 3.65 | 3.20 | 1.29 | 6.22 |
| **36** | 0.40 | 0.40 | 10.00 | 4.01 | 3.56 | 1.39 | 6.91 |
| **48** | 0.53 | 0.54 | 10.00 | 5.37 | 4.92 | 1.68 | 9.56 |
| **54** | 0.52 | 0.53 | 10.00 | 5.28 | 4.83 | 1.66 | 9.38 |

## G2 Growth on Methanol at various concentrations(Figure 4.14)

**Table G2. 1growth on methanol at various concentrations**

| conc | 0 g/L | | | 5 g/L | | |
|---|---|---|---|---|---|---|
| **time** | OD1 | OD2 | AVERAGE | OD1 | OD2 | AVE |
| **0** | 0.401 | 0.402 | 0.402 | 0.562 | 0.568 | 0.565 |
| **12** | 0.297 | 0.281 | 1.445 | 0.285 | 0.271 | 1.39 |
| **16** | 0.341 | 0.338 | 1.698 | 0.324 | 0.315 | 1.5975 |
| **20** | 0.367 | 0.375 | 1.855 | 0.362 | 0.366 | 1.82 |
| **24** | 0.381 | 0.387 | 1.920 | 0.409 | 0.408 | 2.0425 |

Results were obtained when cells were inoculated in chemically defined medium containing methanol at the concentrations listed.

| conc | 7.5 g/L | | | 10 g/L | | |
|---|---|---|---|---|---|---|
| **time** | OD1 | OD2 | AVE | OD1 | OD2 | AVE |
| **0** | 0.484 | 0.475 | 0.4795 | 0.448 | 0.436 | 0.442 |
| **12** | 0.275 | 0.264 | 1.3475 | 0.305 | 0.319 | 1.56 |
| **16** | 0.305 | 0.319 | 1.56 | 0.352 | 0.345 | 1.7425 |
| **20** | 0.337 | 0.339 | 1.69 | 0.384 | 0.381 | 1.9125 |
| **24** | 0.366 | 0.37 | 1.84 | 0.402 | 0.408 | 2.025 |

| time | 30 g/L | | | 40 g/L | | | |
|---|---|---|---|---|---|---|---|
| | OD1 | OD2 | AVE | OD1 | OD2 | AVE | DIL FACTOR |
| **0** | 0.372 | 0.37 | 0.371 | 0.328 | 0.32 | 0.324 | 1 |
| **12** | 0.184 | 0.193 | 0.9425 | 0.187 | 0.18 | 0.9175 | 5 |
| **16** | 0.193 | 0.204 | 0.9925 | 0.197 | 0.192 | 0.9725 | 5 |
| **20** | 0.215 | 0.206 | 1.0525 | 0.201 | 0.202 | 1.0075 | 5 |
| **24** | 0.24 | 0.25 | 1.225 | 0.201 | 0.215 | 1.04 | 5 |

**Table G2. 2 methanol concentrations when cells were grown on methanol alone in chemically defined medium**

| conc | 0 g/L | 5 g/L | 7.5 g/L | 10 g/L |
|---|---|---|---|---|
| **time** | ave CDW (g/L) | ave CDW (g/L) | ave CDW (g/L) | ave CDW (g/L) |
| **1.07** | **1.07** | **0.90** | **0.83** | **1.07** |
| **0.49** | 2.67 | 2.59 | 3.00 | 0.49 |
| **0.58** | 3.07 | 3.00 | 3.36 | 0.58 |
| **0.68** | 3.51 | 3.25 | 3.69 | 0.68 |
| **0.76** | 3.94 | 3.55 | 3.91 | 0.76 |

| 20 g/L | 30 g/L | 40 g/L |
|---|---|---|
| **ave CDW (g/L)** | ave CDW (g/L) | ave CDW (g/L) |
| **0.903696** | 0.688716 | 0.597276 |
| **1.980545** | 1.800584 | 1.751946 |
| **2.744163** | 1.89786 | 1.858949 |
| **3.191634** | 2.014591 | 1.927043 |
| **3.488327** | 2.350195 | 1.990272 |



### G3 Effect of evaporation on methanol concentrations (Figure 4.15)

A 30 g/L methanol solution was prepared in distilled water contained in a shake flask. The flask was incubated under the same conditions of the experimental flasks investigated above. Samples were taken periodically and the methanol concentration measured. The results are shown below.

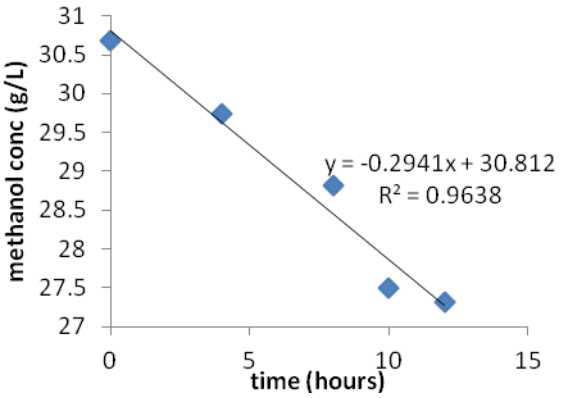


**Figure A. 14 Determination of the methanol evaporation rate.**

The gradient indicates the rate of methanol evaporation.

Thus the hourly rate of methanol evaporation was determined to be 0.294 g/Lh. This value was accounted for in calculating the average methanol concentration in each flask.

### *G 3.1 Methanol evaporation in shake flask and bioreactor experiments*

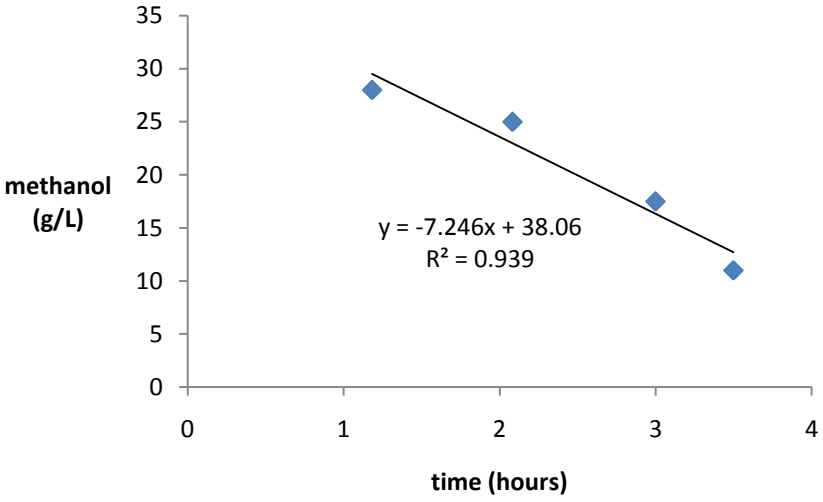


**Figure A. 15Determining methanol utilisation rate in bioreactor.**

From the over-fed methanol, an attempt was made to compute the rate of depletion due to uptake by cells. In order to do this the value computed above needed to account for the rate at which methanol evaporated in a bioreactor. To do this a bioreactor containing 4 L of distilled water with a dilution of methanol at 30 g/L was prepared. The reactor was set to the same conditions as for fed-batch on methanol and the rate of evaporation determined.

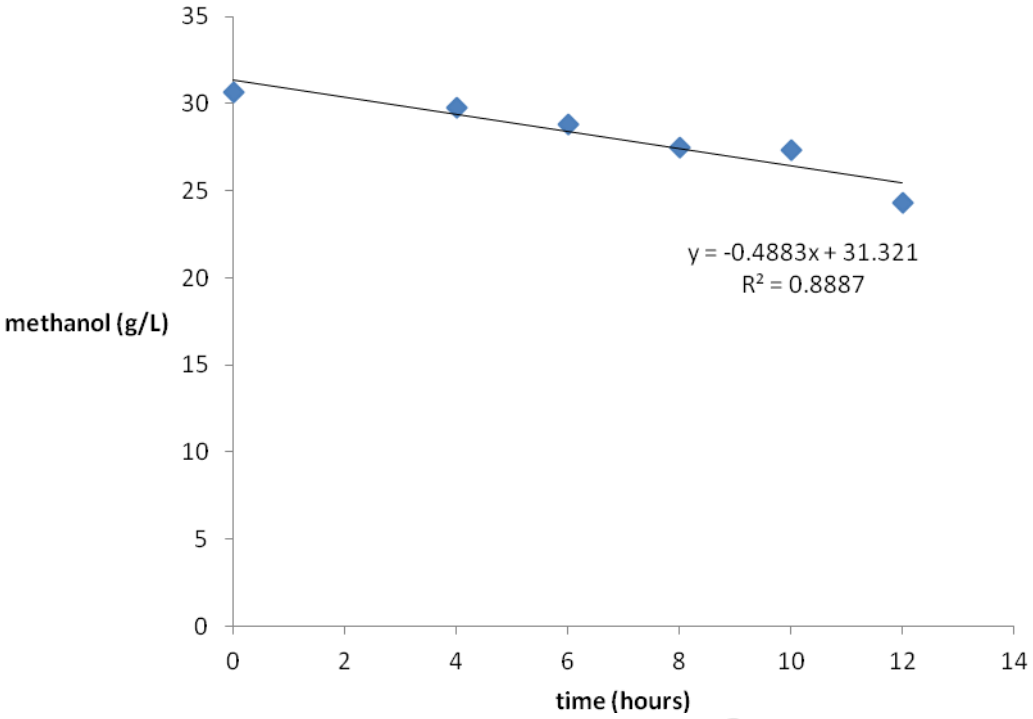


**Figure A. 16The methanol depletion rate was observed to be approximately 0.5 g/Lh**

The methanol feed rate in subsequent experiments was not allowed to exceed 7 g/Lh. Thus based on these data three optimised fed-batch runs were conducted to boost biomass production prior to induction with methanol.

### *G3.2 Effect of Starting Methanol Concentration on Evaporation Rate*

**Table G. 1 Effect of intial methanol concentration on evaporation rate.**

| Sample | Starting Conc (g/L) | Final Conc (g/L) | Duration (h) | Rate of evaporation (g/Lh) |
|---|---|---|---|---|
| 5 | 8 | 5 | 12 | 0.25 |
| 10 | 12 | 9 | 12 | 0.25 |
| 20 | 20 | 16 | 12 | 0.33 |
| 30 | 38 | 34.5 | 12 | 0.29 |
| 40 | 44 | 40 | 12 | 0.33 |
| | | | Average | 0.29 |
| | | | Standard Dev | 0.04 |

Flasks containing distilled water with methanol at 5, 10, 20, 30 and 40 g/L were incubated at experimental conditions and the methanol concentration measured at the start and end of each experiment. The results were used to determine the effect of the initial methanol concentration on the evaporation rate.

## Appendix H Fed-batch experiments

The fed-batch experiments consisted of 3 distinct phases:

- Glycerol batch phase
- Glycerol fed-batch phase
- Methanol induction phase

The glycerol batch phase was usually conducted on 2.5 to 3 L of chemically defined medium containing 50 g/L glycerol. OD and substrate measurements were taken routinely. The OD values were used to compute the cell dry weight.

### H1 Fed batch run 1 Figure 4.20

The results below were obtained during the batch phase on glycerol in Run 1



**Table H1. 1 fedbatch data run 1**

| time | OD1 | OD2 | dil factor | average | ln (OD) | CDW (g/L) |
|---|---|---|---|---|---|---|
| **0** | 0.42 | 0.42 | 1.00 | 0.42 | -0.87 | 0.28 |
| **16** | 0.10 | 0.10 | 100.00 | 10.15 | 2.32 | 7.39 |
| **17** | 0.11 | 0.11 | 100.00 | 10.95 | 2.39 | 9.02 |
| **18** | 0.14 | 0.14 | 100.00 | 13.95 | 2.64 | 11.53 |
| **19** | 0.17 | 0.17 | 100.00 | 17.05 | 2.84 | 13.24 |
| **20** | 0.21 | 0.20 | 100.00 | 20.35 | 3.01 | 16.22 |
| **21** | 0.26 | 0.27 | 100.00 | 26.65 | 3.28 | 20.70 |
| **22** | 0.28 | 0.28 | 100.00 | 28.05 | 3.33 | 25.45 |
| **23** | 0.31 | 0.33 | 100.00 | 32.30 | 3.48 | 26.10 |
| **24** | 0.40 | 0.41 | 100.00 | 40.40 | 3.70 | 26.95 |
| **25** | 0.46 | 0.47 | 100.00 | 46.30 | 3.84 | 30.71 |
| **26** | 0.55 | 0.56 | 100.00 | 55.20 | 4.01 | 37.03 |
| **27** | 0.67 | 0.67 | 100.00 | 67.20 | 4.21 | 44.82 |
| **28** | 0.77 | 0.77 | 100.00 | 77.15 | 4.35 | 51.42 |
| **29** | 0.59 | 0.56 | 200.00 | 114.70 | 4.74 | 76.16 |

The expression used to determine the flow rate is:

$$F = \frac{\mu X V}{Y_{\frac{x}{s}} S_0} e^{\mu(t-t_0)}$$

where F is the flow rate; μ is the desired growth rate, X is the biomass at the end of the batch and the start of the fed-batch; V is the volume at the start of the fed-batch; Yx/s is the yield coefficient obtained on glycerol during the batch (before fed-batch); $S_0$ is the glycerol concentration in the feed (200 g/L); t is the time after the start of fed-batch; $t_0$ is the start time (set to zero). The flow rate

calculated in l/h allows the expected weight of the feed to be determined based on the density of the feed. The actual weight determined by noting the balance measurement on an hourly basis allowed the determination of the actual flow rate, hence the actual volume of the reactor. From this the total cells produced could be calculated (multiply the g/L value by the volume of the reactor).

**Table H1. 2 Use of feed density to maintain exponential flow rate**

| **weight of media + apparatus** | **5.428** | **kg** |
|---|---|---|
| **weight of apparatus** | 1.858 | kg |
| **weight of media** | 3.570 | kg |
| **volume of media** | 3.00 | L |
| **density** | 1.19 | kg/L |

**Table H1. 3 input parameters to determine exponential flow rate**

| | | |
|---|---|---|
| μ | 0.15 | /h |
| X | 26.64 | g/L |
| V | 2.5 | L |
| Yx/s | 0.52 | g/g |
| So | 200 | g/l |



**Table H1. 4actual and projected flow rate values as well as reactor volume.**

| **time** | **constant** | **F(l/h)** | **weight of media +apparatus (expected) (kg)** | **Actual weight (kg)** | **vol added (L)** | **Vol Of Reactor (L)** | **glycerol added (g)** |
|---|---|---|---|---|---|---|---|
| **1** | 0.095 | 0.11 | 5.307 | 5.394 | 0.03 | 2.53 | 6.2 |
| **2** | 0.095 | 0.12 | 5.167 | 5.142 | 0.26 | 2.79 | 52.7 |
| **3** | 0.095 | 0.14 | 5.005 | 5.1064 | 0.30 | 3.09 | 59.2 |
| **4** | 0.095 | 0.17 | 4.816 | 5.0621 | 0.34 | 3.42 | 67.4 |
| **5** | 0.095 | 0.20 | 4.596 | 4.9509 | 0.44 | 3.86 | 87.9 |
| **6** | 0.095 | 0.23 | 4.341 | 4.9455 | 0.44 | 4.31 | 88.9 |
| **7** | 0.095 | 0.27 | 4.045 | 4.8815 | 0.50 | 4.81 | 100.6 |
| | | vol added (theor) | 177.965 | total vol | 2.3 | total | 362.3 |

The flow rate given was computed as indicated previously. Given the density the expected weight of the media and apparatus could be computed. This was compared to the actual value and from this the volume of media added to the reactor computed. The volume added was used to determine the new volume of the reactor during the glycerol fed-batch phase. From this the amount of glycerol added could be determined.

**Table H2. 1 Fedbatch run 2**

| time | OD | OD | dil factor | average | ln (OD) | CDW(g/L) | cdw tot (g) | ln (CDW) | dissolved oxygen (%) | glycerol |
|---|---|---|---|---|---|---|---|---|---|---|
| **0** | 0.418 | 0.420 | 1.000 | 0.419 | -0.870 | 0.278 | 0.835 | -0.181 | 98.3 | 49.900 |
| **14** | 0.100 | 0.103 | 100.000 | 10.150 | 2.317 | 7.390 | 22.170 | 3.099 | 71.500 | 37.800 |
| **15** | 0.110 | 0.109 | 100.000 | 10.950 | 2.393 | 9.015 | 27.045 | 3.298 | 56.600 | 32.500 |
| **16** | 0.138 | 0.141 | 100.000 | 13.950 | 2.635 | 11.530 | 34.590 | 3.544 | 41.400 | 30.100 |
| **17** | 0.169 | 0.172 | 100.000 | 17.050 | 2.836 | 13.240 | 39.720 | 3.682 | 26.500 | 28.700 |
| **18** | 0.209 | 0.198 | 100.000 | 20.350 | 3.013 | 16.220 | 48.660 | 3.885 | 39.100 | 25.900 |
| **19** | 0.262 | 0.271 | 100.000 | 26.650 | 3.283 | 20.700 | 62.100 | 4.129 | 28.400 | 20.100 |
| **20** | 0.282 | 0.279 | 100.000 | 28.050 | 3.334 | 25.450 | 76.350 | 4.335 | 4.500 | 14.200 |
| **21** | 0.314 | 0.332 | 100.000 | 32.300 | 3.475 | 26.100 | 78.300 | 4.361 | 25.300 | 4.900 |
| **22** | 0.403 | 0.405 | 100.000 | 40.400 | 3.699 | 26.950 | 80.850 | 4.393 | 65.000 | 0.000 |
| **23** | 0.459 | 0.467 | 100.000 | 46.300 | 3.835 | 30.711 | 99.743 | 4.603 | 12.200 | 12.200 |
| **24** | 0.547 | 0.557 | 100.000 | 55.200 | 4.011 | 37.028 | 123.051 | 4.813 | 10.800 | 0.000 |
| **25** | 0.673 | 0.671 | 100.000 | 67.200 | 4.208 | 44.819 | 151.805 | 5.023 | 4.200 | 0.000 |
| **26** | 0.771 | 0.772 | 100.000 | 77.150 | 4.346 | 51.421 | 187.278 | 5.233 | 2.000 | 0.000 |
| **40** | 0.585 | 0.562 | 200.000 | 114.700 | 4.742 | 76.161 | | | 78.000 | 0.000 |
| **43** | 0.533 | 0.527 | 200.000 | 106.000 | 4.663 | 70.384 | | | 4.800 | 0.000 |
| **44** | 0.565 | 0.511 | 200.000 | 107.600 | 4.678 | 71.446 | | | 3.500 | 0.000 |
| **45** | 0.544 | 0.545 | 200.000 | 108.900 | 4.690 | 72.310 | | | 2.200 | 0.000 |
| **46** | 0.660 | 0.536 | 200.000 | 119.600 | 4.784 | 79.414 | | | 2.100 | 0.000 |
| **47** | 0.613 | 0.538 | 200.000 | 115.100 | 4.746 | 76.426 | | | 1.200 | 0.000 |
| **48** | 0.697 | 0.677 | 200.000 | 137.400 | 4.923 | 91.234 | | | 1.400 | 0.000 |
| **49** | 0.617 | 0.622 | 200.000 | 123.900 | 4.819 | 82.270 | | | 95.000 | 0.000 |
| **50** | 0.573 | 0.583 | 200.000 | 115.600 | 4.750 | 76.758 | | | 1.400 | 0.000 |
| **51** | 0.577 | 0.566 | 200.000 | 114.300 | 4.739 | 75.895 | | | 1.000 | 0.000 |
| **64** | 0.604 | 0.597 | 200.000 | 120.100 | 4.788 | 79.746 | | | 87.900 | 0.000 |
| **65** | 0.612 | 0.549 | 200.000 | 116.100 | 4.754 | 77.090 | | | 26.000 | 0.000 |
| **66** | 0.598 | 0.62 | 200 | 121.8 | 4.80238 | 80.875 | | | 16 | 0 |
| **70** | 0.58 | 0.549 | 200 | 112.9 | 4.726502 | 74.966 | | | 72.2 | 0 |

## H3 Fed-batch Run 3

**Table H3. 1 Fedbatch data run 3**

| time | OD1 | OD2 | DIL FACTOR | AVERAGE | ln(OD) | CDW(g/L) | time | total cells (g) | Glycerol (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| **0** | 0.49 | 0.49 | 1.00 | 0.49 | -0.71 | 0.33 | 0.00 | | 55.73 |
| **9** | 0.26 | 0.26 | 10.00 | 2.62 | 0.96 | 1.74 | 9.00 | | 43.72 |
| **12** | 0.46 | 0.46 | 10.00 | 4.60 | 1.52 | 3.05 | 12.00 | | 38.73 |
| **14** | 0.66 | 0.67 | 10.00 | 6.66 | 1.89 | 4.42 | 14.00 | | 37.37 |
| **16** | 0.97 | 0.97 | 10.00 | 9.68 | 2.27 | 6.43 | 16.00 | | 36.44 |
| **17** | 0.57 | 0.57 | 20.00 | 11.37 | 2.43 | 7.55 | 17.00 | | 36.00 |
| **18** | 0.70 | 0.71 | 20.00 | 14.12 | 2.64 | 9.38 | 18.00 | | 26.23 |
| **19** | 0.86 | 0.85 | 20.00 | 17.05 | 2.83 | 11.32 | 19.00 | | 20.83 |
| **20** | 0.20 | 0.21 | 100.00 | 20.65 | 3.02 | 13.71 | 20.00 | | 11.41 |
| **21** | 0.24 | 0.25 | 100.00 | 24.25 | 3.18 | 16.10 | 21.00 | | 10.16 |
| **22** | 0.30 | 0.31 | 100.00 | 30.10 | 3.40 | 19.99 | 22.00 | | 0.29 |
| **23** | 0.36 | 0.37 | 100.00 | 36.25 | 3.59 | 24.07 | 23.00 | 60.18 | 0.20 |
| **24** | 0.47 | 0.48 | 100.00 | 47.10 | 3.85 | 33.22 | 24.00 | 85.07 | 0.16 |
| **25** | 0.50 | 0.51 | 100.00 | 50.60 | 3.92 | 35.69 | 25.00 | 91.39 | 0.00 |
| **26** | 0.55 | 0.55 | 100.00 | 54.75 | 4.00 | 38.62 | 26.00 | 102.37 | 0.00 |
| **27** | 0.61 | 0.61 | 100.00 | 60.65 | 4.10 | 42.78 | 27.00 | 120.75 | 0.00 |
| **28** | 0.67 | 0.68 | 100.00 | 67.55 | 4.21 | 47.64 | 28.00 | 145.92 | 0.00 |
| **29** | 0.75 | 0.77 | 100.00 | 75.90 | 4.32 | 53.53 | 29.00 | 177.03 | 0.00 |
| **30** | 0.41 | 0.41 | 200.00 | 81.40 | 4.39 | 57.41 | 30.00 | 208.89 | 0.00 |
| **38** | 0.55 | 0.56 | 200.00 | 111.30 | 4.71 | 78.50 | | | 0.00 |
| **48** | 0.57 | 0.57 | 200.00 | 114.00 | 4.73 | 80.40 | | | 0.00 |
| **56** | 0.55 | 0.56 | 200.00 | 110.80 | 4.70 | 78.15 | | | 0.00 |
| **63** | 0.54 | 0.55 | 200.00 | 109.40 | 4.69 | 77.16 | | | 0.00 |
| **70** | 0.56 | 0.54 | 200.00 | 110.70 | 4.70 | 78.08 | | | 0.00 |
| **74** | 0.55 | 0.59 | 200.00 | 114.00 | 4.73 | 80.40 | | | 0.00 |
| **82** | 0.55 | 0.56 | 200.00 | 110.80 | 4.70 | 78.15 | | | 0.00 |

Shaded region represents glycerol fed-batch phase. Values obtained beyond 38 hours represent methanol induction phase, including methanol concentrations which were found to be zero.

**Table H3. 2 flow rate maintained at exponential rate**

| time | const | F(l/h) | Expected vol | expected weight | actual weight |
|---|---|---|---|---|---|
| **1** | 0.1077 | 0.127 | 2.62 | 2.939 | 3.004 |
| **2** | 0.1077 | 0.151 | 2.77 | 2.916 | 2.975 |
| **3** | 0.1077 | 0.179 | 2.95 | 2.888 | 2.893 |
| **4** | 0.1077 | 0.212 | 3.17 | 2.855 | 2.824 |
| **5** | 0.1077 | 0.251 | 3.42 | 2.816 | 2.82 |
| **6** | 0.1077 | 0.298 | 3.72 | 2.769 | 2.732 |
| **7** | 0.1077 | 0.354 | 4.07 | 2.714 | 2.631 |
| | **vol added** | 1.575 | | 1.503 | |

**Table H3. 3 glycerol addition during exponential feed**

| time | vol added (L) | gly added (g) | actual vol reactor (L) | theor gly added (g) |
|---|---|---|---|---|
| **1** | 0.06 | 12.35 | 2.56 | 25.53 |
| **2** | 0.09 | 18.34 | 2.65 | 30.26 |
| **3** | 0.17 | 34.91 | 2.82 | 35.87 |
| **4** | 0.24 | 48.72 | 3.06 | 42.51 |
| **5** | 0.24 | 49.63 | 3.30 | 50.39 |
| **6** | 0.33 | 67.37 | 3.63 | 59.73 |
| **7** | 0.43 | 87.59 | 4.06 | 70.80 |

## H4 Fed batch Run 4

**Table H4. 1 all data for fedbatch run 4**

| time | OD1 | OD2 | DIL FACTOR | AVERAGE | cdw (g/L) | total cells | glycerol (g/l) |
|---|---|---|---|---|---|---|---|
| **0** | 0.24 | 0.24 | 1.00 | 0.24 | 0.17 | | 53.32 |
| **4** | 0.40 | 0.41 | 2.00 | 0.81 | 0.58 | | 49.09 |
| **9** | 0.18 | 0.18 | 10.00 | 1.76 | 1.23 | | 48.72 |
| **11** | 0.24 | 0.24 | 10.00 | 2.39 | 1.71 | | 40.51 |
| **19** | 0.13 | 0.13 | 100.00 | 12.85 | 9.01 | | 30.22 |
| **20** | 0.16 | 0.16 | 100.00 | 15.90 | 11.26 | | 20.51 |
| **21** | 0.20 | 0.20 | 100.00 | 19.95 | 13.93 | | 12.23 |
| **22** | 0.29 | 0.29 | 100.00 | 29.00 | 16.21 | | 0.00 |
| **23** | 0.38 | 0.38 | 100.00 | 38.00 | 20.53 | | 0.00 |
| **24** | 0.42 | 0.43 | 100.00 | 42.20 | 26.52 | 66.30 | 0.00 |
| **23** | 0.42 | 0.43 | 100.00 | 42.20 | 30.03 | 78.58 | 0.00 |
| **24** | 0.48 | 0.48 | 100.00 | 47.60 | 33.65 | 93.14 | 0.00 |
| **25** | 0.52 | 0.54 | 100.00 | 52.80 | 37.29 | 110.40 | 0.00 |
| **26** | 0.29 | 0.29 | 200.00 | 58.00 | 41.31 | 130.86 | 0.00 |
| **27** | 0.32 | 0.33 | 200.00 | 64.60 | 45.73 | 155.11 | 0.00 |
| **28** | 0.35 | 0.36 | 200.00 | 71.30 | 49.78 | 184.97 | 0.00 |
| **29** | 0.38 | 0.38 | 200.00 | 75.50 | 53.31 | 217.97 | 0.00 |
| **42** | 0.45 | 0.45 | 200.00 | 89.05 | 56.40 | | 0.00 |
| **46** | 0.30 | 0.30 | 300.00 | 90.75 | 57.48 | | 0.00 |
| **53** | 0.31 | 0.31 | 300.00 | 92.48 | 58.57 | | 0.00 |
| **64** | 0.31 | 0.31 | 300.00 | 92.93 | 58.86 | | 0.00 |
| **70** | 0.30 | 0.30 | 300.00 | 89.25 | 56.53 | | 0.00 |
| **82** | 0.30 | 0.31 | 300.00 | 91.80 | 58.15 | | 0.00 |
| **86** | 0.30 | 0.30 | 300.00 | 90.15 | 57.10 | | 0.00 |
| **106** | 0.29 | 0.30 | 300.00 | 88.43 | 56.01 | | 0.00 |
| **110** | 0.30 | 0.29 | 300.00 | 88.88 | 56.29 | | 0.00 |

continuation of Run 4 parameters

| time (hours) | glycerol (g/l) | eppie weight (g) | eppie+ cells (g) | cells (g/ml) | cells (g/L) | reactor vol (L) |
|---|---|---|---|---|---|---|
| **0** | 53.32 | 1.03 | 1.03 | 0.00 | 0.17 | 2.50 |
| **4** | 49.09 | 1.02 | 1.02 | 0.00 | 0.58 | 2.50 |
| **9** | 48.72 | 1.02 | 1.02 | 0.00 | 1.23 | 2.50 |
| **11** | 40.51 | 1.02 | 1.03 | 0.00 | 1.71 | 2.50 |
| **19** | 30.22 | 1.02 | 1.04 | 0.02 | 9.01 | 2.50 |
| **20** | 20.51 | 1.02 | 1.04 | 0.02 | 11.26 | 2.50 |
| **21** | 12.23 | 1.02 | 1.05 | 0.03 | 13.93 | 2.50 |
| **22** | 0.00 | 1.02 | 1.06 | 0.04 | 20.53 | 2.50 |
| **23** | 0.00 | 1.02 | 1.08 | 0.05 | 26.52 | 2.50 |
| **24** | 0.00 | 1.07 | 1.13 | 0.06 | 30.03 | 2.50 |
| **23** | 0.00 | 1.02 | 1.08 | 0.06 | 30.03 | 2.62 |
| **24** | 0.00 | 1.02 | 1.08 | 0.07 | 33.65 | 2.77 |
| **25** | 0.00 | 1.02 | 1.10 | 0.07 | 37.29 | 2.96 |
| **26** | 0.00 | 1.02 | 1.10 | 0.08 | 41.31 | 3.17 |
| **27** | 0.00 | 1.01 | 1.10 | 0.09 | 45.73 | 3.39 |
| **28** | 0.00 | 1.02 | 1.12 | 0.10 | 49.78 | 3.72 |
| **29** | 0.00 | 1.07 | 1.18 | 0.11 | 53.31 | 4.09 |
| **42** | 0.00 | 1.04 | 1.15 | 0.11 | 56.40 | 4.09 |
| **46** | 0.00 | 1.02 | 1.14 | 0.12 | 57.48 | 4.15 |
| **53** | 0.00 | 1.03 | 1.15 | 0.12 | 58.57 | 4.25 |
| **64** | 0.00 | 1.03 | 1.15 | 0.12 | 58.86 | 4.41 |
| **70** | 0.00 | 1.03 | 1.14 | 0.11 | 56.53 | 4.50 |
| **82** | 0.00 | 1.03 | 1.15 | 0.12 | 58.15 | 4.68 |
| **86** | 0.00 | 1.07 | 1.18 | 0.11 | 57.10 | 4.74 |
| **106** | 0.00 | 1.04 | 1.15 | 0.11 | 56.01 | 5.03 |
| **110** | 0.00 | 1.06 | 1.18 | 0.11 | 56.29 | 5.09 |

**Table H4. 2 input parameters for run 4**

| | | |
|---|---|---|
| μ(set) | 0.17 | /h |
| X(end of batch) | 26.5 | g/L |
| V | 2.5 | L |
| Yx/s | 0.3766 | g/g |
| $S_0$ | 200 | g/L |

**Table H4. 3 Fed-batch parameters**

| duration of fedbatch | constant | F (l/h) | theor vol (L) | expected weight(kg) | actual weight |
|---|---|---|---|---|---|
| **1** | 0.15 | 0.18 | 2.68 | 2.89 | 2.95 |
| **2** | 0.15 | 0.21 | 2.89 | 2.68 | 2.80 |
| **3** | 0.15 | 0.25 | 3.14 | 2.43 | 2.61 |
| **4** | 0.15 | 0.30 | 3.43 | 2.14 | 2.40 |
| **5** | 0.15 | 0.35 | 3.78 | 1.80 | 2.18 |
| **6** | 0.15 | 0.41 | 4.20 | 1.38 | 1.86 |
| **7** | 0.15 | 0.49 | 4.69 | 0.90 | 1.49 |

**Table H4. 4 glycerol addition**

| glycerol added (g) | actual vol added (L) | vol reactor (L) | total cells (g) | cells formed /glycerol added | cells formed (g) |
|---|---|---|---|---|---|
| **23.40** | 0.12 | 2.62 | 78.58 | 0.53 | 12.29 |
| **30.24** | 0.15 | 2.77 | 93.14 | 0.48 | 14.56 |
| **38.49** | 0.19 | 2.96 | 110.40 | 0.45 | 17.26 |
| **41.37** | 0.21 | 3.17 | 130.86 | 0.50 | 20.46 |
| **44.89** | 0.22 | 3.39 | 155.11 | 0.54 | 24.25 |
| **64.79** | 0.32 | 3.72 | 184.97 | 0.46 | 29.86 |
| **74.54** | 0.37 | 4.09 | 217.97 | 0.44 | 33.00 |

## H5 Fed batch Run 5

| time | OD1 | OD2 | DIL FACTOR | AVERAGE | CDW(g/L) |
|---|---|---|---|---|---|
| **0** | 0.99 | 0.98 | 1.00 | 0.98 | 0.69 |
| **22** | 0.70 | 0.70 | 50.00 | 34.93 | 24.42 |
| **23** | 0.73 | 0.73 | 50.00 | 36.58 | 25.57 |
| **24** | 0.72 | 0.73 | 50.00 | 36.18 | 25.29 |
| **25** | 0.38 | 0.41 | 100.00 | 39.30 | 27.48 |
| **26** | 0.45 | 0.46 | 100.00 | 45.60 | 31.88 |
| **27** | 0.54 | 0.55 | 100.00 | 54.65 | 38.21 |
| **28** | 0.59 | 0.58 | 100.00 | 58.15 | 40.65 |
| **29** | 0.64 | 0.64 | 100.00 | 63.70 | 44.53 |
| **30** | 0.67 | 0.68 | 100.00 | 67.20 | 46.98 |
| **44** | 0.73 | 0.74 | 100.00 | 73.35 | 51.28 |
| **60** | 0.59 | 0.59 | 200.00 | 117.60 | 45.86 |
| **72** | 0.60 | 0.61 | 200.00 | 121.30 | 47.90 |
| **80** | 0.60 | 0.60 | 200.00 | 119.30 | 48.94 |
| **92** | 0.61 | 0.60 | 200.00 | 121.30 | 48.32 |

**Table H4. 5 parameters**

| | | |
|---|---|---|
| µset | 0.21 | /h |
| X | 24.6 | g/l |
| V | 2.5 | L |
| Yx/s | 0.5 | g/g |
| S0 | 200 | g/L |

| | |
|---|---|
| constant | 0.127 |
| weight of bottle+ media | 2.32kg |
| weight of bottle | 1.08kg |
| weight of media | 1.23kg |
| volume | 1.3L |
| density | 0.952kg/L |



**Table H4. 6 Change in Kinetic Parameters over time**

| Time (hours) | constant | F (l/h) | expected weigh (kg)t | actual weight (kg) | glycerol added (g) |
|---|---|---|---|---|---|
| **1** | 0.12 | 0.16 | 2.17 | 2.20 | 31.25 |
| **2** | 0.13 | 0.19 | 1.99 | 1.98 | 44.74 |
| **3** | 0.13 | 0.24 | 1.76 | 1.68 | 64.27 |
| **4** | 0.13 | 0.29 | 1.48 | 1.59 | 18.23 |
| **5** | 0.13 | 0.36 | 1.13 | 1.02 | 119.55 |
| | | | | total | 278.04 |

| Time (hours) | actual vol added (L) | vol reactor (L) | total cells (g) |
|---|---|---|---|
| 1 | 0.16 | 2.66 | 72.98 |
| 2 | 0.22 | 2.88 | 91.81 |
| 3 | 0.32 | 3.20 | 122.31 |
| 4 | 0.09 | 3.29 | 133.85 |
| 5 | 0.60 | 3.89 | 173.25 |
| | 1.3 | | |

## Appendix I Downstream Approaches to Insulin Purification'

### I1 Affinity Purification via Protino-Ni-TED His-tagged Kit

#### *Product Specifications*

1000* Protino Ni-TED packed columns (250 mg resin) employs gravity flow to purify proteins. Details regarding overall specifications given in kit. The kit contains all the required buffers and columns. However, the buffer composition listed below was used to re-constitute the buffers when the buffers in the kit had been exhausted.

#### *Buffers*

Lysis Equilibration Wash Buffer (LEW buffer, 1 L)

- 50 mM $NaH_2PO_4$
- 300 mMNaCl
- Adjust pH to 8.0 using NaOH

Denaturing Solubilisation Buffer

- 50 mM $NaH_2PO_4$
- 300 mMNaCl
- 8 M Urea
- 250 mM imidazole
- Adjust pH to 8.0 using NaOH



#### *Protocol for Affinity Purification*

1. Harvest extracellular supernatants obtained from bioreactor samples, typically stored in 2 ml Eppendorf tubes at -20$^0$C.
2. Prepare working solutions (re-prepare as necessary)
    a. 1 X LEW Buffer: Mix 2.5 ml of 8 X LEW buffer supplied in kit with 17.5 ml distilled water to get 1 X LEW buffer.
    b. Elution Buffer: Mix 1 ml of 1 X elution buffer with 6 ml distilled water
3. Equilibrate Ni-TED column by addition of 4 ml of LEW buffer. Allow to drain by gravity.
4. Add the extracellular material to the column and allow it to drain by gravity

5. Wash the column twice with 2 x 4 ml volumes of 1 X LEW buffer. Collect each wash in a separate tube.

6. Elute the his-tagged protein using the 1 X Elution buffer, allowing it to drain by gravity, collected in a separate tube.

7. Run each of the fractions on a 15 % SDS- PAGE gel to determine which fraction contains the insulin precursor..

The columns must be washed four times with 4 ml of 70 % Ethanol per wash in order to regenerate the resins. The affinity columns may be stored indefinitely in a 70 % ethanol solution at $4^{o}$C.

## I2 Determination of Protein Concentration

Proteins were quantified according to the Bradford protocol. Pure Bovine Serum Albumin (BSA) protein was used as a standard to determine protein concentration of samples.

1. Prepare a 5 mg/ml stock concentration of BSA by weighing 10 mg of BSA into 2 ml of distilled water.

2. Prepare a series of dilutions to obtain concentrations within the 0- 0.7 mg/ml range (the appropriate range for the samples in this study). Additionally, prepare a control sample containing the colorimetric reagent with no protein added. This serves as a 'blank' and is used to normalise the absorbance values obtained.

3. Measure the absorbance at 504 nm in the Microtitre Plate Reader

.The standard curve of the data in the table are plotted in A 18

**Table I1. 1 Standard Curve of BSA (mg/ml) vs absorbance**

| BSA (mg/ml) | absorbance 1 | absorbance 2 | average | actual |
|---|---|---|---|---|
| **0** | 0.597 | 0.617 | 0.607 | 0 |
| **0.125** | 0.697 | 0.641 | 0.669 | 0.062 |
| **0.25** | 0.748 | 0.746 | 0.747 | 0.14 |
| **0.5** | 0.857 | 0.855 | 0.856 | 0.249 |
| **0.675** | 0.929 | 0.947 | 0.938 | 0.331 |

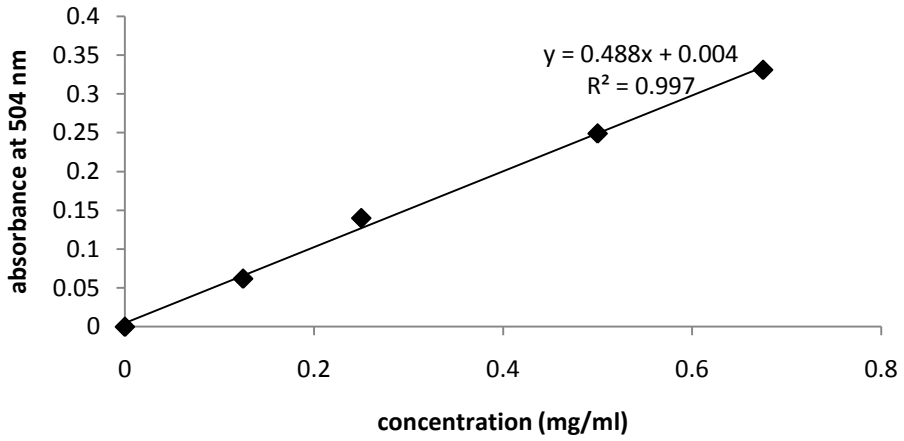


**Figure A. 17 Absorbance at 504 nm vs BSA protein concentration (mg/ml)**

## I3 Western Blot Solutions

The solutions used were prepared fresh at the beginning of the set of experiments and stored at room temperature unless stated otherwise.

**10x Phosphate Buffer Saline (PBS)**

80 g NaCl

2 g KCl

14.4 g $Na_2HPO_4$

2.4 g $KH_2PO_4$

dissolve into 800 ml $dH_2O$.

Adjust pH to 7.4

Make up to 1000 ml

autoclave

make a 1x working solution

**4x Laemmli Buffer**

4.4 ml 0.5 M Tris (pH 6.8)

4.4 ml Glycerol

2.2 ml 20% SDS

0.5 ml 1% Bromophenol Blue

0.5 ml Beta-Mercapto-Ethanol

Aliquot and store at -20$^0$C.

Dilute to 2x before use.

**10x Running Buffer**

30.3 g (0.25 M) Tris Base

144 g (1.92 M) Glycine 10 g (1%) SDS or appropriate for concentrated stock; qs 1000 ml dH2O ;Dilute 1:10 with dH2O. pH will be 8.3

**10x Towbin's Electrotransfer Buffer**

30.3 g Tris Base

144 g Glycine

Make up to 1000 ml $dH_2O$

**1x Towbin's**

100 ml 10x stock

200 ml (20%) Methanol

Make up to 1000 ml $dH_2O$

### I4 SDS-PAGE Gel and Western Blot

Run the gel according to the instructions below.

1. Measure protein concentration in duplicate using Bradford using a BSA standard

curve. Run up to 70 µg/lane otherwise protein overloading results.

2. Mix extract 1:1 with Laemmli buffer and heat to 80-100$^0$C for 5 min (excess boiling may destroy protein).

3. Run on an SDS-PAGE 15 % composition until the blue front is at the bottom of the gel (gel preparation given below).

Bio-Rad Mini-Gel Box Running Conditions: 75 V x 3 hrs (dye about in the bottom of the gel) 4. Transfer (only for Insulin Precursor):

Blot onto a nitrocellulose membrane. Pre-wet materials in transfer buffer. Stack in the following order:

• case (clear side)

• sponge

• Whatman paper

• membrane

• gel

• Whatman paper

• sponge

• case (black side)

Place in the transfer apparatus with black side facing black).

Bio-Rad Mini-Gel Box Electrotransfer:

• 90 V x 1 hr (use ice-pack to cool down the apparatus)

• 70 V x 2 hr (use ice-pack to cool down the apparatus)

Transfer at 0.5 A-Hour (0.5 A for one hour, or 0.05 A for 10 hrs.)

4. Block the membrane for 30 min in 20-30 ml 1x PBS + 5% non-fat dry milk + 0.1% Tween 20, in a small dish on an orbital shaker.

5. Incubate with primary antibody diluted in 2 ml 1x PBS + 5% milk + 0.1% Tween 20. Incubate o/n at $4^0$C

6. Wash 3 x for 5-10 min in ~50 ml 1x PBS + 0.1 % Tween 20 at RT in a small dish on shaker.

7. Incubate with secondary antibody for 30 min to 1 hr at RT in 2 ml 1x PBS + 5% milk + 0.1% Tween 20 on shaker

8. Wash 3 x 10 min each in ~50 ml 1x PBS + 0.1% Tween 20 at RT in a small dish on a shaker.

11. Rinse with d $H_2$ O. Place nitrocellulose membranes in X-ray cassette.

12. Detect protein with ECL kit (2 ml/membrane). In a separate tube, mix black and white ECL solutions in a 1:1 ratio. Aliquot solution onto membranes and wait for 1 minute. Drain the ECL, wrap in plastic and expose to film.

***Gel Recipe***

The SDS PAGE gel is made up of the resolving gel and the stacking gel. The compositions are given in the table below. The percentages refer to the 'thickness' of the gels: increasing thickness of the gel implies smaller pores.

| Resolving Gel (30 ml) | 6 % | 8 % | 10 % | 12 % | 15 % |
|---|---|---|---|---|---|
| Distilled water | 16.2 | 14.2 | 12.2 | 10.2 | 7.2 |
| 30 % acrylamide mix | 6 | 8 | 10 | 12 | 15 |
| 4 x resolving-gel buffer | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| 20 % SDS | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |

| | | | | | |
|---|---|---|---|---|---|
| 20 % APS | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| TEMED | 0.024 | 0.018 | 0.012 | 0.012 | 0.012 |

**Stacking Gel Mixture**

| | |
|---|---|
| Water | 6.9 ml |
| 30 % acrylamide mix | 1.7 ml |
| 8 x stacking buffer | 1.25 ml |
| 20 % SDS | 0.05 ml |
| 20 % APS | 0.05 ml |
| TEMED | 0.01 ml |



**Staining Solution**

To make up staining solution, dissolve 0.25 g of Coomassie Brilliant Blue in a solution made up of 45 %(v/v) water, 45 % (v/v) methanol and 10 % (v/v) acetic acid. Upon dissolving, filter the solution before use.

**Destaining Solution**

To make up de-stain solution, make up a 12.5 % (v/v) isopropanol, 10 % (v/v) glacial acetic acid solution in distilled water.